

**ASSESSMENT OF THE FLAME ANGELFISH (*Centropyge loriculus*)
AS A MODEL SPECIES IN STUDIES ON EGG AND LARVAL QUALITY
IN MARINE FISHES**

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Thesis Advisors: Dr. David W. Townsend and Dr. Linda J. Kling

An Abstract of the Thesis Presented
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This project sought to determine if flame angelfish (*Centropyge loriculus*) could serve as models in examination of environmental and dietary effects on egg quality in marine fishes. Evaluation of 21 marine ornamental species identified flame angelfish as being amenable to egg quality research, due to their rapid conditioning and frequency of spawning. At the onset of this project, accidental copper introduction to broodstock systems required assays to determine the effects of copper exposure on survival and reproduction. Flame angelfish exhibited acute sensitivity to copper, as 60% of fish exposed to 0.25mg/L died within 12 hours of exposure. Likewise, fish exposed to 0.20 and 0.15mg/L exhibited 40% mortality within 48 hours. Furthermore, copper at 0.10mg/L significantly reduced fecundity and negatively affected embryonic development among orchid dottyback (*Pseudochromis fridmani*) broodstock.

A series of experiments was conducted to determine the effects of water chemistry and broodstock diet on flame angelfish reproduction, as well as to compile baseline spawning performance and egg quality data for this species. Results revealed that water chemistry significantly affected spawning performance, as fish maintained in sterilized ocean water exhibited greater fecundity, egg fertilization rates and egg viability than pairs held in water from saltwater wells. However, sterilization of ocean water by chlorine at levels >25ppm significantly reduced fecundity and egg fertilization.

Flame angelfish readily adapted to a variety of formulated feeds and results from current experiments demonstrated that maternal diet significantly affected egg quality. Fish that were fed a diet containing 3.63% n-3 highly unsaturated fatty acids (HUFA) exhibited significantly greater fecundity, fertilization rates and egg viability than fish that were fed diets with lower n-3 HUFA levels. Furthermore, over-all egg quality, egg and larval size metrics, and survival to yolk-exhaustion, were not significantly different between fish fed the High n-3 and Control diets. Daily egg production from 18 pairs was recorded over a 20-month period and averaged 1,000-1,500 eggs per female. Mean daily egg fertilization rates ranged from 60-80% and hatch rates were normally 80%. Egg quality characteristics responded to maternal dietary changes within weeks, indicating that experiments of shorter duration than those currently reported may be possible.

DEDICATION

This dissertation is dedicated in loving memory

of my sister Jaclyn Rose Callan.

She inspired me to do my best at all times
and always made me laugh, even when I didn't feel like it.

She remains in my heart forever.

This is for you Jackie.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
LIST OF TABLES	xiii
LIST OF FIGURES	xv

Chapter

1. INTRODUCTION	1
1.1. The Marine Aquarium Industry	1
1.2. Angelfish Taxonomy, Distribution and Reproductive Biology	4
1.2.1. Reproductive Biology	6
1.2.2. Spawning Rituals	7
1.2.3. Angelfish Eggs and Larvae	10
1.2.4. Angelfish Culture	12
1.3. Broodstock Nutrition	14
1.3.1. Lipids	16
1.3.2. Studies on n-3 HUFA	18
1.3.3. Importance of ARA	23
1.3.4. Vitamins	27
1.3.5. Carotenoids	28
1.3.6. Nutrition and Coral Reef Fishes	29
1.4. Summary	31
1.5. Current Research	32
1.6. References	34

2.	DEVELOPMENT OF A MARINE ORNAMENTAL FISH BROODSTOCK LABORATORY FOR USE IN YEAR-ROUND AQUACULTURE RESEARCH	43
2.1.	Abstract	43
2.2.	Introduction	44
2.3.	Methods	45
2.3.1.	System Design and Configuration	45
2.3.2.	Acquisition of Marine Ornamental Fish Broodstock	51
2.3.2.1.	Demersal Species	51
2.3.2.2.	Pelagic Species	52
2.3.3.	Broodstock Conditioning.....	53
2.3.3.1.	Clownfish, Dottybacks and Other Demersal Species	53
2.3.3.2.	Angelfish and Other Pelagic Spawning Species	55
2.4.	Results	56
2.4.1.	Clownfish and Dottyback Spawning Behavior	56
2.4.2.	Angelfish Spawning Behavior	59
2.4.3.	Egg Collector Modification	60
2.5.	Discussion	66
2.6.	Acknowledgments	68
2.6.	References	69
3.	EFFECTS OF COPPER EXPOSURE ON MARINE ORNAMENTAL FISH REPRODUCTION AND SURVIVAL	70
3.1.	Abstract	70

3.2. Introduction	71
3.3. Methods	75
3.3.1. Experiments 1 and 2: Effects of Copper Exposure on Survival of the Flame Angelfish (<i>Centropyge lorciulus</i>)	75
3.3.1.1. Experiment 1	75
3.3.1.2. Experiment 2	76
3.3.2. Experiment 3: Effects of Copper Exposure on Reproduction and Embryonic Development of the Orchid Dottyback (<i>Pseudochromis fridmani</i>)	77
3.3.2.1. Experimental Conditions and Broodstock Husbandry	77
3.3.2.2. Copper Treatment	78
3.3.2.3. Sampling Procedures	78
3.3.3. Statistical Analysis	81
3.4. Results	81
3.4.1. Experiment 1	81
3.4.2. Experiment 2	84
3.4.3. Experiment 3	86
3.5. Discussion	91
3.6. Acknowledgments	95
3.7. References	97

4.	DETERMINATION OF THE EFFECTS OF WATER SOURCE AND WATER TREATMENT ON FLAME ANGELFISH (<i>Centropyge loriculus</i>) REPRODUCTION	99
4.1.	Abstract	99
4.2.	Introduction	100
4.3.	Methods	104
4.3.1.	System Configuration	104
4.3.2.	Fish Acquisition, Quarantine and Conditioning	106
4.3.3.	Ocean Water Sterilization Protocol	108
4.3.4.	Experiment Stocking and Environmental Conditions	108
4.3.5.	Broodstock Husbandry	109
4.3.6.	Egg Collection and Sampling.....	110
4.3.7.	Statistical Analysis	111
4.4.	Results	111
4.4.1.	Effects of Ocean Water Chlorination Protocol on Flame Angelfish Egg Fertilization	111
4.4.2.	Effects of Water Source on Fecundity	113
4.4.3.	Effects of Water Source on Egg Fertilization	114
4.4.4.	Effects of Water Source on Egg Viability	115
4.4.5.	Effects of Treatment of OI Water on Flame Angelfish Reproduction	117
4.4.5.1.	Effects of Water Treatment on Fecundity	117

4.4.5.2. Effects of Water Treatment on Fertilization Rate	118
4.4.5.3. Effects of Water Treatment on Egg Viability	119
4.5. Discussion	120
4.6. Acknowledgments	123
4.7. References	125
5. DESCRIPTION OF THE EFFECTS OF BROODSTOCK DIET ON EGG QUALITY PARAMETERS AND SUSTAINED SPAWNING CHARACTERISTICS IN FLAME ANGELFISH (<i>Centropyge loriculus</i>)	126
5.1. Abstract	126
5.2. Introduction	127
5.3. Methods	129
5.3.1. Experiment Stocking and Environmental Conditions	129
5.3.2. Broodstock Husbandry	131
5.3.2.1. Feeding	131
5.3.2.2. Weight and Length Measurements	133
5.3.2.3. Tank Maintenance	134
5.3.2.4. Egg Collection and Sampling	135
5.3.3. Egg Fatty Acid Analysis.....	137
5.3.4. Determination of Optimal Environment for Assessing Day 3 Survival	138
5.3.5. Statistical Analysis	139

5.4. Results	139
5.4.1. Flame Angelfish Size at Maturity	139
5.4.2. Effects of Diet on Flame Angelfish Egg Production and Egg Quality	140
5.4.3. Diet and Egg Biochemistry	145
5.4.4. Flame Angelfish Daily Egg Production	149
5.4.5. Flame Angelfish Egg Hatch Rate and Larval Survival to Yolk-Exhaustion	150
5.5. Discussion	151
5.6. Acknowledgments	157
5.7. References	158
6. STUDIES ON THE EFFECTS OF DIETARY n-HUFA LEVEL ON FLAME ANGELFISH (<i>Centropyge loriculus</i>) EGG AND LARVAL QUALITY	161
6.1. Abstract	161
6.2. Introduction	162
6.3. Methods	164
6.3.1. Experiment 1: Effects of Dietary n-3 HUFA Level On Flame Angelfish Egg and Larval Quality	164
6.3.1.1. Pair Ranking and Assignment to Treatment Groups	164
6.3.1.2. Experiment Stocking and Environmental Conditions	164
6.3.1.3. Diet Formulation	165
6.3.1.4. Diet Processing	167

6.3.1.5. Feeding	168
6.3.1.6. Tank Maintenance	170
6.3.1.7. Egg Collection and Sampling	170
6.3.1.8. Egg Measurements and Hatch Rate Determination	172
6.3.1.8.1. Egg Measurements	172
6.3.1.8.2. Hatch Rate Determination	172
6.3.1.8.3. Day 3 Survival Determination	173
6.3.2. Experiment 2: Effects of High Dietary n-3 HUFA Level On Flame Angelfish Egg Quality	173
6.3.2.1. Pair Ranking and Assignment to Treatment Groups	173
6.3.2.2. Broodstock Husbandry	173
6.3.2.2.1. Feeding	173
6.3.2.2.2. Diet Formulation and Processing	174
6.3.2.3. Fatty Acid Analysis of Diets	176
6.3.3. Statistical Analysis	176
6.4. Results	177
6.4.1. Experiment 1	177
6.4.1.1. Fatty Acid Analysis	177
6.4.1.2. Effects of Diet on Egg Quality	179
6.4.1.2.1. Effects of Diet on Egg Biochemistry	179
6.4.1.2.2. Effects of Diet on Fecundity	179
6.4.1.2.3. Effects on Egg Fertilization	180

6.4.1.2.4. Effects on Egg Viability	182
6.4.1.2.5. Effects on Egg Size, Percent Hatch, Larval Size and Percent Survival	183
6.4.2. Experiment 2	184
6.4.2.1. Fatty Acid Composition of Diets	184
6.4.2.2. Effects on Fecundity	186
6.4.2.3. Effects on Fertilization	187
6.4.2.3. Effects on Egg Viability	188
6.5. Discussion	189
6.6. Acknowledgments	196
6.7. References	197
7. SIGNIFICANCE AND FUTURE WORK	199
BIBLIOGRAPHY	201
APPENDIX. Fatty Acid Profiles of Flame Angelfish Diets and Eggs	212
BIOGRAPHY OF THE AUTHOR	217

LIST OF TABLES

Table 2.1.	Spawning data from observed marine ornamental species	64
Table 3.1.	Mean concentration of copper exposure per treatment and percent survival of flame angelfish following 48 hours of copper exposure during Experiment 1	82
Table 3.2.	Summary of spawning data collected from orchid dottyback broodstock over the duration of Experiment 3	87
Table 4.1.	Composition of “Raw” control diet used for marine ornamentals at the Oceanic Institute	110
Table 5.1.	Composition and proximate analyses of experimental diets formulated for flame angelfish and a commercially available aquarium diet	132
Table 5.2.	Composition of “Raw” control diet used for marine ornamentals at the Oceanic Institute	133
Table 5.3.	Fatty acid composition of experimental diets for flame angelfish broodstock	147
Table 5.4.	Mean fatty acid composition of total lipids in eggs from flame angelfish broodstock fed different diets	148
Table 6.1.	Composition of formulated diets fed to flame angelfish broodstock in Experiment 1	169
Table 6.2.	Calculated proximate composition and n-3 HUFA levels of treatment diets from Experiment 1	170

Table 6.3.	Composition of formulated diets fed to flame angelfish broodstock in Experiment 2	175
Table 6.4.	Calculated composition and HUFA levels of treatment diets from Experiment 2	176
Table 6.5.	Fatty acid composition of treatment diets in Experiment 1	178
Table 6.6.	Composition and HUFA levels of Control diet and formulated treatment diets from Experiment 1	179
Table 6.7.	Flame angelfish mean egg and larval size characteristics, percent hatch and percent survival of larvae to day 3 post-hatch from broodstock fed different diets in Experiment 1	183
Table 6.8.	Fatty acid composition of treatment diets in Experiment 2	185
Table 6.9.	Composition and n-3 HUFA levels of Control diet and formulated treatment diets from Experiment 2	186
Table A.1.	Complete fatty acid profiles of flame angelfish broodstock diets tested in Chapter 5	213
Table A.2.	Complete fatty acid profiles of eggs from flame angelfish broodstock fed different diets in Chapter 5	214
Table A.3.	Complete fatty acid profiles of flame angelfish broodstock diets tested in Chapter 6 (Exp. 1)	215
Table A.4.	Complete fatty acid profiles of flame angelfish broodstock diets tested in Chapter 6 (Exp. 2)	216

LIST OF FIGURES

Figure 1.1.	Photographs of flame angelfish egg and larva	4
Figure 1.2.	Photograph of a male flame angelfish (<i>C. loriculus</i>)	5
Figure 1.3.	Characteristic stages found in <i>Centropyge</i> courtship and spawning ritual	9
Figure 2.1.	Photographs of the glass aquaria incorporated into the marine ornamental broodstock laboratory at the Aquaculture Research Center (ARC) at The University of Maine	46
Figure 2.2.	Photographs of the polyethylene tanks incorporated into the marine ornamental broodstock laboratory at the ARC	46
Figure 2.3.	Photograph of the filtration equipment for the marine ornamental broodstock system at the ARC	48
Figure 2.4.	Schematic diagram of the marine ornamental broodstock holding system at the ARC	50
Figure 2.5.	Photographs of male and female flame angelfish	52
Figure 2.6.	Photograph of Common clownfish (<i>Amphrion ocellaris</i>) tending to their eggs on the side of a flowerpot	54
Figure 2.7.	Photograph of an Orchid dottedback (<i>Pseudochromis fridmani</i>) pair and their PVC “den.”	55
Figure 2.8.	Photograph of the pelagic tank configuration used for spawning flame angelfish at the ARC	56

Figure 2.9.	Schematic diagrams of angelfish holding tanks and egg collectors at the ARC	61
Figure 2.10.	Photograph of modified egg collector used for concentrating flame angelfish eggs	62
Figure 2.11.	Top view of modified egg collector showing very little disturbance of water in the collector, despite high flow-rates through the unit	63
Figure 3.1.	Photograph of the bioassay system at the Oceanic Institute	76
Figure 3.2.	Photomicrograph series depicting the normal embryonic development of the orchid dottyback (<i>Pseudochromis fridmani</i>)	80
Figure 3.3.	Schematic diagram of aquarium used to hatch dottyback eggs	81
Figure 3.4.	Survival of flame angelfish exposed to six levels of copper for 48 hours	83
Figure 3.5.	Relationship of flame angelfish survival to increased copper for 48 hours of exposure	84
Figure 3.6.	Recorded copper levels during trial Experiment 2	85
Figure 3.7.	Survival of the flame angelfish exposed to copper for 168 hours	86
Figure 3.8.	(A) Mean number of eggs and hatched larvae produced and (B) mean percent normal embryos at 24 and 72 hours post-fertilization, before, during and after copper treatment	88
Figure 3.9.	Photographs of abnormalities in early embryonic development of orchid dottyback eggs exposed to copper	89

Figure 3.10.	Photographs of abnormalities in late embryonic development of orchid dottyback eggs exposed to copper	90
Figure 4.1.	Photograph of a portion of the flame angelfish broodstock system at the Oceanic Institute	101
Figure 4.2.	Mean fertilization rates of flame angelfish eggs from January 2004 through December 2005 (n=12 pairs)	102
Figure 4.3.	Flame angelfish total monthly egg production for 2004 and 2005 (n=12 pairs).....	103
Figure 4.4.	Photograph of a portion of the Indoor Marine Ornamental (IMO) system at the Oceanic Institute	104
Figure 4.5.	Photograph of the Indoor Marine Ornamental (IMO) filtration system at the Oceanic Institute	105
Figure 4.6.	Schematic diagram of modified Indoor Marine Ornamental (IMO) system tanks at the Oceanic Institute	106
Figure 4.7.	Photographs of representative egg quality categories for flame angelfish eggs	111
Figure 4.8.	Flame angelfish weekly mean fertilization rates while held in ocean water treated with different levels of chlorine	112
Figure 4.9.	Flame angelfish mean fecundity (number of eggs per spawn) while held in OI water vs. sterilized ocean water	114
Figure 4.10.	Flame angelfish mean fertilization rates while held in OI water vs. sterilized ocean water	115

Figure 4.11.	Flame angelfish mean egg viability while held in OI water vs. sterilized ocean water	116
Figure 4.12.	Flame angelfish mean egg production in OI water treated by recirculation (OI-RC) vs. untreated OI water (OIW)	117
Figure 4.13.	Flame angelfish weekly mean egg fertilization rates in OI water treated by water recirculation (OI-RC) vs. untreated OI water (OIW)	118
Figure 4.14.	Flame angelfish weekly mean egg viability in OI water treated by water recirculation (OI-RC vs. untreated OI water (OIW)	119
Figure 5.1.	Photograph of a portion of the flame angelfish broodstock system at the Oceanic Institute	130
Figure 5.2.	Photograph of a flame angelfish near the PVC scaffolding used as an artificial “reef.”	134
Figure 5.3.	Photographs of tank surface drain emptying into egg collector (top) and close-up of water entering egg collector with screen up (bottom)	136
Figure 5.4.	Photographs of representative egg quality categories for flame angelfish eggs	137
Figure 5.5.	Length (TL) and weight of sexually mature male and female flame angelfish (n=36 individuals)	140
Figure 5.6.	Flame angelfish mean daily spawn size on different diets over the duration of the 7-month feeding experiment (n=4 pairs)	141

Figure 5.7.	Flame angelfish mean egg fertilization and egg viability rates on different diets over the duration of the 7-month feeding experiment (n=4 pairs).....	142
Figure 5.8.	Flame angelfish mean monthly egg production for September 2005 at the conclusion of 7-month diet trial (n=4 pairs)	143
Figure 5.9.	Flame angelfish mean monthly egg production after one month of feeding on a “mixed” diet (n=4 pairs)	144
Figure 5.10.	Flame angelfish mean monthly egg production after four months of feeding on a “mixed” diet (n=4 pairs)	145
Figure 5.11.	Relative fatty acid profiles of flame angelfish diets (A) and viable (buoyant) eggs (B) during feeding experiment	149
Figure 5.12.	Flame angelfish mean daily egg production from October 2005 through May 2007 (n=18 pairs)	150
Figure 5.13.	Flame angelfish mean egg hatching and survival of pre-feeding larvae to 72h in aerated vs. non-aerated culture tanks (n=4 tanks)	151
Figure 6.1.	Photograph of modified egg collector with screen slid into place to concentrate eggs	171
Figure 6.2.	Flame angelfish mean daily egg production during Experiment 1	180
Figure 6.3.	Flame angelfish mean daily egg fertilization rates during Experiment 1	181

Figure 6.4.	Flame angelfish mean daily egg viability rates during Experiment 1	182
Figure 6.5.	Flame angelfish weekly mean fecundity from Experiment 2	187
Figure 6.6.	Flame angelfish weekly mean egg fertilization rates from Experiment 2	188
Figure 6.7.	Flame angelfish weekly mean egg viability rates from Experiment 2	189
Figure 6.8.	Flame angelfish mean egg fertilization rate of control treatment pairs (n=4 pairs) and combined (all treatments including control) mean (n=18 pairs) from August 2005 to May 2007	190
Figure 6.9.	Flame angelfish mean egg viability rate of control treatment pairs (n=4) and combined treatments mean (n=18) from August 2005 to May 2007	191

Chapter 1

INTRODUCTION

1.1. The Marine Aquarium Industry

The ornamental fish trade is a rapidly growing industry worldwide (Andrews, 1990). It is estimated that global trade in aquarium fish and related products is worth over 3 billion dollars annually (Friedlander, 2001; Olivier, 2001). The estimated retail value for the U.S. aquarium industry including accessories and livestock is now more than 1 billion dollars. Approximately 1.5 to 2 million people worldwide (50% USA, 25% EU and 25% other) keep marine aquaria (Green, 2003). The United States market (60% of world market) is now greater than 250 million dollars annually for marine species alone (Ounaies, 1998; Larkin and Degner, 2001). An estimated 30 million tropical marine fish comprising over 1,000 species are harvested from coral reefs annually. Of these, between 8 and 15 million are sold in the United States every year (Green, 2003).

The main source of marine aquarium fish is from wild collections on coral reefs. The majority of exports into the aquarium trade are from developing nations such as the Philippines, Indonesia, Singapore and Sri Lanka (Corbin et al., 2003; Olivier, 2001). Unfortunately, many methods utilized for collections have been ecologically unsound, and in most cases, are not sustainable (Rubec, et al., 2001; Friedlander, 2001). Therefore, the increasing demand for rare, high quality reef specimens is taking an even greater toll on already stressed coral reef ecosystems (Wood, 2001). In addition to the realized environmental impacts, the typical extended-chain of custody associated with wild collection also adversely affects the quality of fish available to the end consumer.

Moreover, the declining condition of coral reefs has caused increased regulation on any activities that impact the reef environment; this includes the restriction, and in some cases banning, of collection of ornamental fish (Wood, 2001).

In order to help preserve the reef environment and to continue to supply a growing market, alternative sources of marine ornamental fish must be found. A recent industry survey reported that most marine hobbyists (65%) buy cultured marine organisms when available and 73% felt that cultured organisms were necessary for the future of the hobby (Moe, 2001). Additionally, most consumers (90%) said they would pay more for fish that were certified “reef friendly” or captive raised. Therefore, culture of marine ornamental species could help to meet this increasing demand, while reducing pressure on coral reefs. The problem remains, however, that the culture of marine ornamental species is in its infancy and to date has met with many challenges.

Despite reports of large numbers of marine ornamental species having been spawned in captivity, few of these species have been successfully reared through late developmental stages (Holt, 2003). To date, only 21 species of marine ornamentals, belonging to 4 different families, are reared at a commercial level (Moe, 1997 & 2001; Tlusty, 2002). These 21 species make up less than 5% of all marine species traded (Moe, 1999). Thus, the industry is relying almost completely (~95%) on wild collection to sustain itself. This is in strong contrast to the freshwater ornamental industry where over 90% of all species sold are cultured (Olivier, 2003; Wood, 2001).

Several factors are responsible for the difficulties encountered in earlier attempts to culture marine ornamentals. Chief among them is the difficulty in feeding the small (<2.0 mm) pelagic larvae. All of the currently mass-cultured species, such as anemone

fish, spawn demersal eggs, which produce relatively large ($>3.0\text{mm}$) larvae. Such larvae can be reared using conventional “food-fish” methods (rotifers and *Artemia sp.* as live prey at first feeding) with a high degree of success. However, these same culture methods have proven ineffective with most coral reef species, as the majority of these fish produce larvae that are too small to initially feed on rotifers or *Artemia sp.* Therefore, the early life stages remain a critical bottleneck in the production of most marine ornamental species (Ostrowski and Laidley, 2001; Holt, 2003).

Marine angelfish of the genus *Centropyge* may serve as an ideal model for research attempts to overcome the current culture constraints and furthering the successful development of marine ornamental aquaculture. Angelfish have been successfully maintained in aquaria for decades and some species have spawned and been reared in captivity. Angelfish produce small ($<800\mu\text{m}$), pelagic eggs (representative of many coral reef species) that hatch in less than 18 hours at 26°C (Fig. 1.1a). The resulting larvae are small ($<2.0\text{mm}$) and share many of the same early larval characteristics as most coral reef species of commercial interest (Fig. 1.1b). Another, particularly advantageous, characteristic is that many *Centropyge* species will spawn daily, providing a continuous supply of eggs and larvae. The research potential of utilizing this constant supply of eggs and larvae could advance efforts to overcome current bottlenecks and facilitate the development of culture methods that could then be applied to other species.

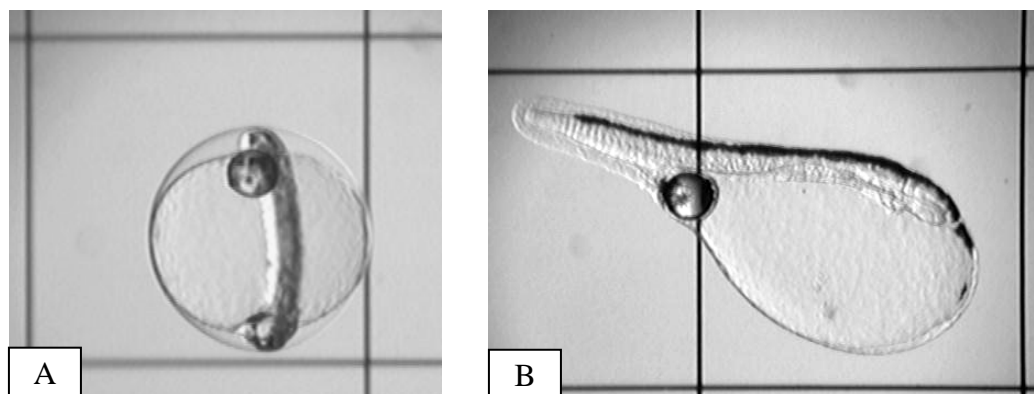


Figure 1.1. Photographs of flame angelfish egg and larva. (A) Flame angelfish egg 16h post-fertilization, nearing hatch. (B) Flame angelfish larva at hatch. Box grid on slide = 1mm.

1.2 Angelfish Taxonomy, Distribution and Reproductive Biology

Marine angelfish comprise the family Pomacanthidae, in which there are 88 recognized species, divided into 8 genera: *Centropyge*, *Chaetodontoplus*, *Euxiphipops*, *Genicanthus*, *Holocanthus*, *Paracentropyge*, *Pomacanthus* and *Pygoplites* (Delbelius et al., 2003). The Pomacanthidae are part of the larger order of Perciformes, which includes most coral reef fishes. The family exhibits circumtropical distribution, although the majority of species occur on shallow reefs (<30m) in areas of coral, sponge or rocky substrate (Thresher, 1984; Delbelius et al., 2003). Delbelius et al., (2003) describes angelfish as having continuous dorsal fins and ovate to rhomboid shaped bodies, which are covered in small scales. These fish have small mouths containing many tricuspid teeth, which are used for grazing algae, sponges or small benthic invertebrates. All angelfish feature a large and distinctive, backward-pointing spine which protrudes from their gill plate. This “cheek spine” is a diagnostic for all the species, and is present even in early juvenile stages (Delbelius et al., 2003).

The genus *Centropyge* (Kaup, 1860) has the most species: 32 species divided into three subgenera and six species complexes (Pyle, 2003). The “*bispinosa*” complex of the genus *Centropyge* includes five species (*C. bispinosa*, *C. ferrugata*, *C. loriculus*, *C. potteri*, and *C. shepardi*) (Shultz et al., 2007). The flame angelfish (Fig. 1.2), *C. loriculus* (Günther, 1874), is easily recognized by its bright orange to red coloration and normally has several vertical black stripes on its body, although the amount of black stripes can vary geographically (Delbelius, 2003; Shultz et al., 2007). This species is found in the central to west Pacific, ranging from eastern Indonesia to the Marquesas Islands. This species is common in Palau, the Marianas, Marshalls and Society Islands. The majority of exports of this species into the marine aquarium trade originate from the Marshall Islands and Christmas Island (Delbelius et al., 2003).



Figure 1.2. Photograph of a male flame angelfish (*C. loriculus*).

1.2.1. Reproductive Biology

Most of what is known about the reproductive biology of angelfish stems from captive (laboratory) study of the smaller “pygmy” angelfishes of the genus *Centropyge* (Hioki and Suzuki, 1987; Hioki et al., 1990; Sakai et al., 2003; Olivotto et al., 2006) and little has been reported on the reproduction of many of the larger angelfish species (Bauer and Bauer, 1981). However, Thresher (1984) reviewed the biology and ecology of these larger species, showing many apparent similarities in reproduction (pair spawning only, courtship spawning rituals and pelagic release of eggs) among genera.

Field studies on genera *Holocanthus* and *Pomacanthus* by Moyer et al., (1983) and Thresher (1982) respectively, have revealed that in all species observed, courtship and spawning occur at dusk, are preceded by male display to the female, and are not clearly associated with lunar periodicity. Although species differ in their spawning ascent heights and specific details of courtship behavior, all species observed exhibited similar spawning characteristics which culminate in the release of gametes into the water column. Field data collected on *Centropyge* by Bauer and Bauer (1981) and Sakai and Kohda (1997) has further corroborated previously reported laboratory findings indicating that most species within the family share similar reproductive biological traits.

It is thought that most or all of the species of *Centropyge* are protogynous (female to male) hermaphrodites (Thresher, 1984). Males of this genus are larger than females, ranging in size from 10 to 15cm (total length) while females generally remain smaller than 13cm. While it is currently unknown if all angelfish are sequential hermaphrodites, observed sexual dimorphism is common throughout the family, where males tend to be significantly larger than females (Moyer et al., 1983).

The angelfish social system is generally characterized by male dominance and defense of a “harem” of females. The territory size and number of females in the group may vary among species, (Bauer and Bauer, 1981; Sakai and Kohda, 1997; Baensch, 2002) but typically ranges from one to four females. In *Centropyge*, sex change appears to be socially controlled in a manner similar to that of many other reef fish species, such as wrasses (Thresher, 1984). The removal of the male will occasionally result in the takeover of the harem by another male, but usually triggers the largest, most dominant female to change sex to male.

Thresher (1984) reports that sexual transition can be rapid, and within 20 days the ex-female can perform as a fully functional male. This sex reversal is possible due to the presence of thickened, hollow areas of an external membrane of the gonads, which are surrounded by inactive spermatogonia that begin to proliferate upon sex reversal. The thickened areas of membrane expand and differentiate, while the ovaries collapse and degenerate (Thresher, 1984). Interestingly, in at least one species of angelfish, (*Centropyge ferrugata*) sex reversed males can change back to females when placed in the presence of a larger, more dominant male (Sakai et al., 2003). It is currently unknown whether other *Centropyge* species maintain this ability to return to females.

1.2.2. Spawning Rituals

Probably the most comprehensive study to date of *Centropyge* reproduction was reported by Bauer and Bauer (1981), who described the spawning behaviors of six species of *Centropyge* from laboratory observations over a period of 4 to 7 years. Through their research, some important and unique characteristics of this genus emerged.

They demonstrated that: 1) *Centropyge* engage in a complex, daily crepuscular spawning ritual, which nearly always results in spawning; 2) Each female spawns once daily with the harem male; 3) Each female spawns a moderate number of eggs (100's to 1,000) per day, throughout the year. Furthermore, in three of the tropical species studied, spawning occurred throughout the year, with no observable lunar periodicity. There was the first evidence of a dramatically different spawning strategy from most other coral reef fish, of which many exhibit strong spawning seasonality and lunar periodicity.

Bauer and Bauer (1981) reported a consistent pattern of spawning in all species of *Centropyge* observed in aquarium and field observations. Courtship activities were initiated about 1½ hours before the lights went off (by automatic timers) and became more intense prior to culminating in the release of gametes. In the field, they observed a similar pattern where courtship began at dusk and spawning occurred at sunset. They concluded that the spawning behavior of all the species they observed was nearly identical and showed great similarity between the lab and the reef settings. The complex spawning ritual of *Centropyge* was described in detail in by Bauer and Bauer (1981) and is summarized by their figure illustrating the various components of the ritual (Fig. 1.3).

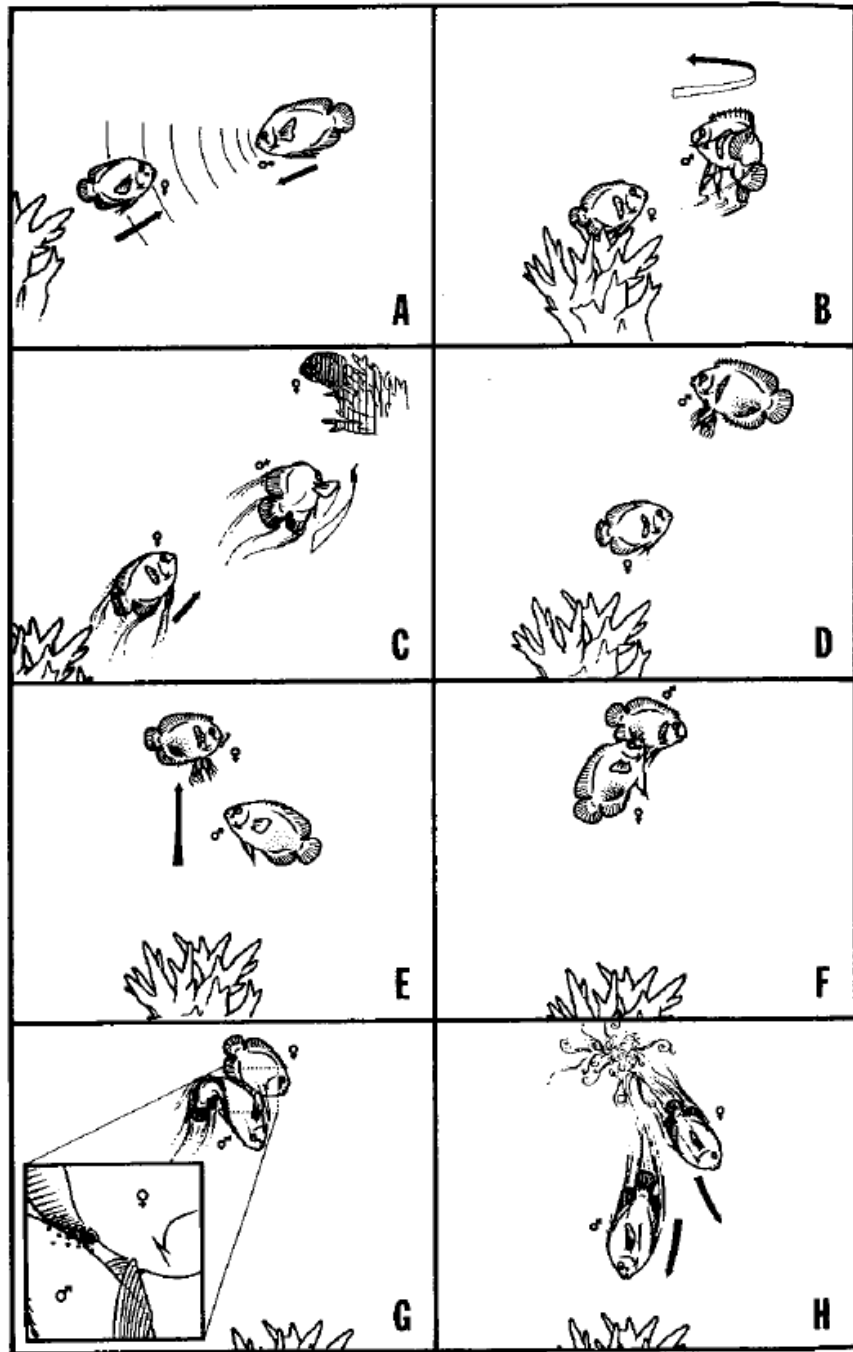


Figure 1.3. Characteristic stages found in *Centropyge* courtship and spawning ritual. (A) Approach-Meet-Grunt (B) Circling (C) Female Follow (D) Quiver Display (E) Flutter Rise (F) Spawning Posture (G) Thrust and Turn (H) Break and Rise. Reproduced with permission from Bulletin of Marine Science. Original figure from: Bauer, J.A, Bauer, S.E., 1981. Reproductive biology of pigmy angelfishes of the Genus *Centropyge* (Pomacanthidae). Bulletin of Marine Science 31, (3) 495-513.

1.2.3. Angelfish Eggs and Larvae

To date, there have only been a few published accounts of the development of eggs and larvae of non-*Centropyge* species. Fujita and Mito (1960) described the egg development and early larval characteristics of *Chaetodonoplus septentrionalis*; Moe (1976, 1977) described the development (egg to juvenile) of *Pomacanthus arcuatus*; Suzuki et al., (1979) described the egg and larval characteristics of *Genicanthus lamark* and *G. semifasciatus*; and Hioki et al., (1982) described the egg and larval development of *Genicanthus melanospilos*. This paucity of literature is likely due to the difficulty of maintaining the larger bodied angelfish genera in captivity for periods long enough to condition them for spawning. Additionally, providing the correct environment to facilitate spawning may also be more difficult to replicate in these species.

Contrary to other angelfish species, information on the embryonic and larval development of *Centropyge* species is plentiful (Hioki and Suzuki, 1987; Hioki et al., 1990; Baensch, 2003; Sakai et al., 2003; Baensch, 2006; Olivotto et al., 2006; Rhody, 2006). Baensch (2006) described the development from egg through juvenile stages of seven species of *Centropyge* (*C. fisheri*, *C. loriculus*, *C. flavissima*, *C. multicolor*, *C. interrupta*, *C. resplendens*, and *C. colini*) in addition to *Paracentropyge multifasciatus*. This work, in addition to others reported for *Centropyge*, has revealed that egg and larval characteristics are very similar among species. Additionally, it appears that most egg and early larval traits are similar across most angelfish genera as well (Hioki and Suzuki, 1987).

Fertilized eggs of *Centropyge* are buoyant, transparent, colorless, spherical, non-adhesive, have a narrow perivitelline space and contain a single oil globule (Hioki et al.,

1990). The reported size range for *Centropyge* eggs varies from 0.65 to 0.75mm and eggs typically hatch after 14-16h post-fertilization at 27°C (Baensch, 2003). Newly hatched larvae have been reported to range in size from 1.3mm for *C. ferrugatus* (Hioki et al., 1990) to 2.3mm for *C. flavissimus* (Olivotto et al., 2006). Rhody (2006) reported that flame angelfish (*C. loriculus*) larvae on average measured 1.12mm in length at hatch and grew to 2.22 mm by 32h post-hatch. This rapid growth during the first day of development likely explains some of the reported variance in size at hatch of *Centropyge*. Baensch (2003) reports that in the three species studied (*C. fisheri*, *C. flavissima*, and *C. loriculus*), size at 5h post-hatch was approximately 1.9mm for all species.

Larval characteristics of *Centropyge* also indicate many similarities among species. Descriptions of *C. ferrugatus* (Hioki et al., 1990) and *C. interrupta* (Hioki and Suzuki, 1987) match very closely the descriptions of *C. fisheri*, *C. loriculus*, *C. flavissima*, *C. multicolor*, *C. interrupta*, *C. resplendens*, and *C. colini* (Baensch, 2006). Generally, larvae are characterized as having a large, ellipsoid yolk sac that extends anteriorly beyond the tip of the head. A single oil globule is present at the posterior portion of the yolk, just anterior of the anus. Larvae generally contain numerous branched melanophores along the dorsal axis of the body and on the surface of the oil globule, although this pigmentation can vary among species. Newly hatched larvae are poorly developed, lacking functional eyes, jaws or alimentary tract. However, development is rapid and by 72h post-hatch the larva has fully developed eyes and digestive tract and is ready to initiate exogenous feeding (Rhody, 2006).

Angelfish are known to have a relatively long and complex pelagic larval stage with durations of up to 6 weeks (Thresher, 1985). Growth and development through the larval stage appears to be similar among species, although time to settlement and metamorphosis can range from approximately 44 days in *Paracentropyge multifasciatus* to 110 days in *Centropyge loriculus* (Baensch, 2006). Baensch (2003, 2006) reported that the average duration from egg to juvenile for *Centropyge* is approximately 60 days. Baensch (2003) reported that F1 generation *C. fisheri* were reproductively mature and spawning at less than 1 year of age.

1.2.4. Angelfish Culture

The only non-*Centropyge* angelfish successfully cultured through juvenile stages are *Pomacanthus arcuatus* (Moe, 1976) and *Genicanthus personatus* by the Waikiki Aquarium in Honolulu, Hawaii in 2002 (Delbelius et al., 2003). Additionally, *Pomacanthus maculosus* are purportedly being cultured in Taiwan (Delbelius et al., 2003). Baensch (2006) successfully reared many *Centropyge* species (*C. fisheri*, *C. loriculus*, *C. flavissima*, *C. multicolor*, *C. interrupta*, *C. resplendens*, and *C. colini*) in addition to *Paracentropyge multifasciatus* from egg through juvenile stages. However, in all of the above citations, no descriptions of rearing methods were published. Olivotto et al. (2006) described the early larval rearing of *C. flavissima*, but unfortunately were not able to rear the larvae through to metamorphosis.

Although *some* broodstock husbandry recommendations have been made regarding the culture of *Centropyge* species (Baensch, 2003), the “secrets” behind the successful rearing through metamorphosis have yet to be published. What has been reported is that for successful spawning to occur, these fish must be provided with the following: 1) 2-3 daily feedings of a high quality diet; 2) long day length (14-16 h light); 3) warm water temperatures (26 -28°C); 4) an adequate size tank (> 300L) with at least 50cm of water depth to allow for spawning rise; and, 5) excellent water quality (Baensch, 2003).

Live zooplankton are presumed to be required for feeding the tiny larvae and, to date, finding appropriate zooplankton prey has been the focus of most larval rearing efforts. Poor success in the culture of species new to aquaculture (including angelfish species) has been mostly attributed to the limited availability of small (<50 micron), highly nutritious and easily cultured prey items (Ostrowski and Laidley, 2001). Recently, much effort has been made to overcome this primary “bottleneck” through the identification and mass-culture of suitable live prey (mostly varied species of copepod nauplii) for the difficult first feeding stages of small marine fish larvae (Stottrup, 2000; McKinnon et al., 2003; Shields et al., 2005). Although there has been some limited success rearing angelfish using both wild-collected and cultured zooplankton, little consideration has been given to the initial “quality” of the pre-feeding larvae, and in particular, very little attention has been given to the influence of broodstock nutrition on the quality of the eggs and larvae being produced. Also, to what degree the effects of parental condition are carried over to the first-feeding larvae are still unknown. Therefore, an important assumption underlying my thesis is that broodstock husbandry

and nutrition may contribute significantly to the current “bottlenecks” experienced by culturists and that such might be a productive avenue of research.

1.3. Broodstock Nutrition

Despite its fundamental importance in aquaculture, broodstock nutrition is one of the least studied and least understood aspects of fish nutrition (Izquierdo et al., 2001). This is due, in part, to the tremendous costs associated with maintaining replicated groups of large, adult fish for necessary study durations that often span periods of years to decades. However, despite these obstacles, it is widely agreed that many of the difficulties encountered in the early larval rearing phases can be attributed to the condition (largely controlled by diet) of the parent broodstock (Rainuzzo et al., 1997).

Field data from McCormick (2003) corroborate earlier studies conducted under culture conditions and strongly support the contention that maternal history influences larval survival as mediated by biochemical products incorporated into the egg during gametogenesis. McCormick (2003) reported that initial larval size, yolk-sac volume, and oil globule size are all affected by “maternal quality” and are predictors indicative of larval fitness and thus survival. In particular, yolk-sac size has been thought to be of fundamental importance, as it serves as the primary source of nutrition for fish larvae as they develop. Larvae with larger yolk reserves are thought to be more capable of surviving suboptimal conditions, such as protracted periods of limited prey availability, which might otherwise reduce growth or increase mortality (Kerrigan, 1997).

Berkeley et al. (2004) reported that in black rockfish (*Sebastes melanopus*) the larval trait most highly correlated with larval performance was the initial volume of the oil globule. Their results revealed that larvae with larger oil globules grew significantly faster and had survival rates twice those of cohorts with the smallest oil globules. These authors further demonstrated that oil globule size was positively correlated with maternal age ($r^2 = 0.82$; $p < 0.0001$). Therefore, small differences in early larval life history traits, in addition to maternal age, may affect future growth and survival of the larvae.

There is also a growing body of evidence to support the hypothesis that early larval characteristics, such as size at hatch and pelagic-larval growth rates, directly contribute to the success of later juvenile settlement (Vigliola and Meekan, 2002; Shima and Findlay, 2002; Bergenius et al., 2002). Also, much of the variability demonstrated during these early life history stages is largely dependant on parental contributions to the eggs (Kerrigan, 1997; Mc Cormick, 2003). Therefore, greater understanding of these contributions is necessary, under captive-culture conditions, in order to ensure the highest quality eggs and larvae are being produced.

It has been demonstrated that, under culture conditions, improvement in nutrition and feeding of broodstock has positive effects on egg quality and seed production in numerous fish species (reviewed by Izquierdo et al., 2001). Conversely, varying egg quality has continued to be one of the most important limiting factors controlling the successful mass production of marine fish (Kjorsvik et al., 1990). Poor egg quality can contribute to reduced larval survival during the known “critical period” of transitioning from endogenous yolk-reserves to exogenous prey capture (Rainuzzo et al., 1997).

However, despite its recognized importance, the problems of egg quality, until recently, have received little attention.

Part of the difficulty in studying egg quality has been a lack of agreement regarding methods used for the assessment of characteristics and metrics of quality (Bromage, 1994). Also, methods to be used in commercial aquaculture applications need to be relatively simple and should be capable of being carried out early in development to avoid wasting hatchery resources. The most commonly used criteria for assessing egg quality are outlined by Kjorsvik et al. (1990). These authors discuss the following characteristics: 1) fertilization processes, in particular fertilization rate; 2) morphology of the earliest cells (blastomeres); 3) egg size; 4) chemical content; and, 5) chromosomal aberrations. However, of the above characteristics, only blastomere morphology has been directly correlated to egg survival as a reliable early indicator of egg quality (Shields et al., 1997). It is also common for characteristics such as fecundity, egg buoyancy, hatching rate, larval survival and “normality” of the survived larvae at the end of endogenous feeding to be considered indicative of overall egg quality (Fernandez-Palacios et al., 1995), but very few studies have correlated these egg characteristics to survivability or overall “fitness” of the larvae.

1.3.1 Lipids

Manipulation of broodstock diet can directly affect gonadal growth and fecundity and dietary lipids are the chemical components that have the greatest effect on the composition of the egg (Rainuzzo et al., 1997). Dietary lipids are sources of metabolic energy and essential fatty acids needed for growth and survival. Moreover, the dietary

essentiality of the n-3 highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid (22:6n-3; DHA) and eicosapentaenoic acid (20:5n-3; EPA) for marine fish are known (Sargent et al., 1999). Unlike freshwater fish species, marine fish require HUFA of the n-3 series such as EPA and DHA due to their inability to synthesize them de-novo, or through elongation and de-saturation of shorter chain fatty acids. Bell and Sargent (2003) explain that marine fish lack either the C₁₈ to C₂₀ elongase multienzyme complex, or the Δ 5-fatty acid desaturase enzyme necessary to convert 18:3n-3 to EPA or 18:2n-6 to arachidonic acid (20:4n-6; ARA). Therefore, these long-chain HUFA are essential fatty acids for marine fish.

Highly unsaturated fatty acids are important structural and physiological components of cell membranes and affect membrane characteristics such as permeability and fluidity (Li et al., 2005), are vital for maintenance of membrane function (Bruce et al., 1999) and have been shown to improve stress resistance in larval and juvenile fish (Furuita et al., 2003). In contrast to mammalian cells, which contain predominantly n-6 ARA as their major HUFA, marine fish contain large amounts of n-3 DHA and EPA in their cellular membranes (Bruce et al., 1999). DHA is found in very high concentrations in neural and visual cell membranes, particularly in the rod cells and synaptosomal membranes (Sargent et al., 1999). Therefore, Sargent et al. (1999) concluded that deficiency of DHA during development could lead to impaired visual and neural function, ultimately affecting numerous physiological and behavioral processes. McEvoy et al. (1998) provided evidence to support this, indicating the importance of DHA and EPA in flatfish pigmentation. They concluded that commonly observed post-larval pigmentation irregularities were directly related to abnormal processing of visual signals

either in the eye or, subsequently, in the brain. These pigmentation abnormalities were corrected, or substantially improved, by the addition of DHA to the larvae's live feeds (Sargent et al., 1999).

1.3.2. Studies on n-3 HUFA

In marine aquaculture, there have been numerous studies linking n-3 HUFA level in broodstock diets to egg and larval quality. In particular, deficiency in n-3 HUFA has been linked to reduced fecundity and decreased fertilization, hatching and viability of marine fish eggs (Rainuzzo et al., 1997). Recently, it has been suggested that excess n-3 HUFA can also be deleterious to egg quality (Fernandez-Palacios et al., 1995; Furita et al., 2002; Li et al., 2005). However, careful examination of these results and other published accounts indicates that optimal dietary n-3 HUFA values may vary among species (Furita et al., 2002; Li et al., 2005).

Fernandez-Palacios et al. (1995) investigated the effects of varying n-3 HUFA levels in diets fed to gilthead sea bream (*Sparus aurata*). In their experiment, diets were developed to contain 1.13%, 1.60%, 2.18% and 3.15% n-3 HUFA (by dry weight). The levels of n-3 HUFA were manipulated through the addition of sardine oil and beef tallow to allow for similar crude lipid levels. Their diets were roughly 50% protein and 15% lipid, and were fed to the broodstock for 3 months.

Their results indicated increased egg viability, percentage of normal eggs, and fertilization rates with increasing n-3 HUFA level. However, fecundity and number of surviving 3 day-old larvae was highest in the treatment receiving the 1.60% diet. They also observed decreased fecundity and yolk-sac hypertrophy in newly hatched larvae

from adults receiving the highest (3.15%) HUFA diet. The authors concluded that spawning quality of gilthead sea bream was positively affected by increasing n-3 HUFA level (up to 1.60%), and that their results agreed with earlier results reported by Wantanabe et al. (1984a,b, c). They further concluded that the egg n-3 HUFA level showed positive correlation with dietary n-3 HUFA level, primarily due to increasing EPA. It is important to note that these authors did not keep the ratios of DHA:EPA:ARA constant in their diets. These ratios have been purported to be as (or more) important as the absolute levels (Sargent et al., 1999).

Additional evidence of the importance of these ratios was reported by Bruce et al. (1999) for European sea bass (*Dicentrarchus labrax*). In those experiments, tuna orbital oil (TOO) was used to replace standard fish oil in one of the two test diets. TOO is much higher in DHA (27%) and ARA (1.8%) than most fish oils and is much lower in EPA (5.4%). The use of this oil allowed for manipulation of DHA and ARA specifically and resulted in ratios much closer to that of the raw fish “standard” diet. The resulting experimental diets were approximately 50% protein and 20% lipid. However, the TOO diet had a higher level of total n-3 HUFA (~5%) compared to the control diet (~3%), mostly due to higher DHA. The TOO diet also had a higher DHA:EPA ratio of 3.5 compared to the control diet of 1.3, as well as a much lower ratio of EPA:ARA of 4 compared to the control diet (14.5).

Their results demonstrated that the TOO diet treatment group had significantly greater percent viability, percent hatching, and larval percent survival to 48 hours than the control diet. The authors attributed the increase in egg quality to the increased amount of DHA and ARA relative to EPA in the TOO diet. Furthermore, their results showed

that ARA was preferentially concentrated in the phosphatidylinositol (PI) lipid class. They concluded that the importance of PI in cell membrane signal transduction, combined with this preferential sequestering of ARA, implies a positive relationship between the amount of ARA in the broodstock diets and egg performance.

Furuita et al. (2000) investigated the effects of n-3 HUFA level on reproductive performance and egg and larval quality of Japanese flounder (*Paralichthys olivaceus*). In their study, diets with similar proximate composition (56% protein, 17% lipid) but varying n-3 HUFA levels (0.4%, 0.8% and 2.1%) were fed to broodstock. As reported by Fernandez-Palacios et al. (1995) ratios between DHA, EPA and ARA were not conserved, and were quite different between diets. In all treatment diets, the DHA:EPA ratio was <1 and the EPA:ARA ratios were >20:1. Egg production, egg buoyancy, fertilization rate, hatching rate, normality of larvae, survival to day 3 post-hatch and survival activity index were all measured for each treatment group.

Their results showed no significant effect of n-3 HUFA level on fecundity, egg buoyancy, or hatching rate. However, as total n-3 HUFA level increased, the percentage of normal larvae increased from 62.6% to 76.9%, survival of the hatched larvae to day 3 was significantly increased from 67.8% to 94.1% and the survival activity index was significantly increased from 15.3 to 30.9. The authors concluded that deficiency in n-3 HUFA level led to decreased egg and larval quality in this species. Their results led to an additional investigation with the same species testing the effects of higher n-3 HUFA level on the same egg and larval characteristics (Furuita et al., 2002).

In their study, Furuita et al. (2002) tested three experimental diets of similar proximate values (52% protein and 15% lipid) on Japanese flounder (*Paralichthys olivaceus*). The diets were formulated to contain increasing quantities of total n-3 HUFA resulting in diets containing total n-3 HUFA levels of 2.1%, 4.8% and 6.2% of the diet dry weight. DHA:EPA ratios were somewhat conserved, ranging from 1.2, 1.6 and 1.5 in diets 1, 2 and 3 respectively. However, EPA:ARA ratios were not conserved and varied from 20:1 in diets 1 and 2 to 30:1 in diet 3. The objective of this study was to determine a suitable upper limit of n-3 HUFA level in diets for Japanese flounder.

Their results showed an increasing, but not significant, trend in fecundity with increasing n-3 HUFA level. However, egg buoyancy decreased significantly from 61.9% to 44.3%, hatching rates decreased significantly from 90.4% to 73.2%, and normality of the hatched larvae decreased significantly from 74.5% to 47.8% with increasing n-3 HUFA level. Similar, but not significant, trends were observed in day 3 survival of larvae and the survival activity index. Another interesting result of this trial is that although diet 3 contained nearly twice the amount of ARA than diet 1, egg ARA content was significantly less in this treatment group than in eggs from the other diet treatments. The authors concluded that the high EPA:ARA ratio (30:1) in this diet may have inhibited ARA incorporation into the egg and thus reduced overall egg quality. They further concluded that a total n-3 HUFA level of 2.1% of dry weight was optimal for Japanese flounder, as the higher levels tested resulted in decreased egg and larval quality.

Although much of the literature over the past twenty years concerning broodstock nutrition has focused on temperate species, recent studies on a few tropical species support that the importance of dietary n-3 HUFA level in egg quality may be universal

among marine fishes. Li et al. (2005) conducted a study on the crescent sweetlips (*Plectorynchus cinctus*), which is an economically important marine finfish in China. They formulated four diets of similar proximate composition (52% protein, 19% lipid) and varied n-3 HUFA content of 1.12%, 2.40%, 3.70% and 5.85% respectively. These diets were compared to a wet-fish “control” diet, which contained an n-3 HUFA level of 3.70%.

Similar to published accounts in other species, their results supported the notion that either deficiency or excess of total n-3 HUFA could negatively affect egg quality. Fecundity and hatch rate was greatest in the control diet, however it was not significantly greater than that of diets 2 (2.4% n-3 HUFA) and 3 (3.70% n-3 HUFA). Fish fed diets 1 and 4 exhibited reduced fecundity and egg buoyancy, but hatch rates and abnormal larval rates did not differ significantly among all treatments. Fish that were fed diet 1 exhibited significantly reduced larval survival of 46% compared to approximately 75% in the other treatments. Larval length was also significantly greater in fish fed diets 2 and 3 and did not differ significantly from the control diet.

These results appear to agree with early studies and indicate that n-3 HUFA level may be important in determining some egg and larval quality characteristics. Not surprisingly, the optimal dietary amounts of n-3 HUFA appear to vary among species. Li et al. (2005) suggest roughly 2.4-3.7% dietary n-3 HUFA for crescent sweetlips, which is higher than the 1.5-2.1% reported for sparids and Japanese flounder (Fururita et al., 2002; Izquierdo et al., 2001) but similar to the 3.5% level reported to allow for good spawning performance in striped jack (Vassallo-Agius et al., 2001). It also appears that marine fish

are very sensitive to the n-3 HUFA level, as dietary changes of only a percent or two can induce substantive improvements in egg quality and/or larval performance.

1.3.3. Importance of ARA

In addition to absolute requirements of n-3 series HUFA for marine fish, Sargent et al. (1999) proposed that the n-6 series HUFA ARA is also an essential fatty acid for marine fish. The authors reported that ARA is the major precursor for eicosanoids, such as 2-series prostaglandins and 4-series leukotrienes in fish and in mammals. Furthermore, eicosanoids have important roles such as signal transductions in neural tissues. Tocher (2003) reviewed the roles of dietary fatty acids and further explained the functions of eicosanoids. Eicosanoids are described as autocrines, or hormone-like compounds, acting in the immediate vicinity of the cells in which they are produced and are responsible for stimulating many of the intermediate chemical reactions involved in reproduction and in egg maturation processes. They also act in vital systemic processes such as blood clotting, immune response, inflammatory response and neural function.

Sargent et al. (1999) reported that ARA and EPA compete for the enzymes cyclooxygenases and lipoxygenases that produce, respectively, 2-series prostanoids and 4-series leukotrienes from ARA and 3-series prostanoids and 5-series leukotrienes from EPA. The eicosanoids produced from ARA are known to be more biologically active, and are inhibited in the presence of excess EPA. Further, Sargent et al. (1999) proposed that both the concentration and ratio of all three essential HUFA, DHA, EPA and ARA were important in marine larval fish nutrition. They proposed an “optimum” ratio of DHA:EPA to be 2:1 (approximately the ratio found in most marine fish eggs) and

DHA:EPA:ARA to be 10:5:1. Despite the important functions of ARA, its effects in fish reproduction and egg quality have, until recent, been little investigated (Bell and Sargent, 2003).

Recently, dietary ARA has also been shown to affect egg and larval quality, juvenile fish growth and adult fish reproduction (reviewed by Bell and Sargent, 2003). These authors reported that inclusion of ARA (up to 0.78% of dry weight) in juvenile turbot (*Scophthalmus maximus*) diets as the only HUFA (purified ARA added to a mixture of hydrogenated coconut oil and oleic acid) resulted in better growth and survival compared to diets that contained mixtures of ARA and DHA or DHA alone. Bell and Sargent (2003) further described that ARA is also critically involved in regulation of the immune function in numerous animal species. More specifically, ARA has a key role as a precursor in the production of prostaglandins (PGE₂) that are produced by monocytes and macrophages. Modulation of PGE₂ levels help to regulate normal immune function and T-cell differentiation. These authors stressed consideration of ARA to be important in future diet studies, particularly in broodstock nutrition research.

Additional evidence to support the importance of ARA in broodstock diet can be found in a study done by Mazorra et al. (2003). These authors investigated the effects of dietary DHA, EPA and ARA on Atlantic halibut (*Hippoglossus hippoglossus*) fecundity, blastomere morphology, egg fertilization and hatch rates. In one of their experiments, two formulated diets were tested on halibut broodstock. The “standard” diet, previously found to perform similar to a diet composed of raw fish, was compared to an “enhanced ARA” diet (containing 1.8% ARA of lipid). Both diets (approximately 61% protein and 16% lipid) contained the same amounts of DHA and EPA (10.9% and 8.5% of total lipid

respectively) but each varied in its ARA content. The standard diet contained 0.06% ARA by diet dry weight and the enhanced diet contained 0.3% ARA by diet dry weight. The resulting EPA:ARA ratios were 21.5 and 4.6 in the standard and enhanced diets respectively.

Mazorra et al. (2003) demonstrated that fish receiving the ARA enhanced diet had higher relative fecundity and significantly higher ($p < 0.05$) fertilization rates, blastomere morphology score, and hatching rates than fish receiving the standard diet. These authors also reported that the ARA levels in the eggs from fish fed the enhanced diet were significantly higher (nearly twice the concentration) than in eggs produced on the standard diet. They concluded that dietary ARA up to 1.8% of the lipid (0.3% of dry weight) had significant beneficial effects on halibut spawning performance and egg and larval quality.

The results of Mazorra et al. (2003) agree with data reported by Furuita et al. (2003), which show that inclusion of ARA up to 0.6% of the diet dry weight had significant positive effects on egg and larval quality of Japanese flounder (*Paralichthys olivaceus*). In their study, three experimental diets (approximately 52% protein, 16% lipid) were developed and tested. Each diet had similar n-3 HUFA levels (2.6% of dry weight) and constant DHA and EPA levels (9.5% and 5% of lipid respectively) thus giving similar DHA:EPA ratios in all diets of approximately 2:1. ARA was tested at 0.1%, 0.6% and 1.2% of diet dry weight, resulting in EPA:ARA ratios of 8.5, 1.4 and 0.67 in diets 1, 2 and 3, respectively.

The results of Furuita et al. (2003) show that fish receiving the 0.6% diet had significantly greater (nearly two-fold) egg production, and that the eggs produced by those fish had significantly higher hatch rates (79.4% vs. 50.6% and 27% in diets 1 and 3, respectively). Also, the 0.6% diet produced significantly more “normal” larvae, which had significantly higher survival to day 3 post-hatch (79.3% vs. 47% and 0% in diets 1 and 3, respectively) and higher survival activity index. Their results also indicated that ARA was incorporated into the eggs at significantly increasing levels with increasing dietary ARA level. These authors concluded that addition of ARA to diets of Japanese flounder improved egg quality, however excess ARA decreased egg quality.

Emata et al. (2003) suggested that ARA might be more important for egg and larval development in tropical species than in temperate and cold-water species. They reported that the overall fatty acid profiles of tropical species appear to show intermediate to high levels of DHA and ARA and low EPA. This results in higher ARA:EPA and DHA:EPA ratios compared to most species studied to date. They reported ARA:EPA ratios in the eggs and larvae of mangrove red snapper (*Lutjanus argentimaculatus*) they studied to be 1.1 and 1.4 respectively. These ratios are much higher than 0.1 found in cod and herring eggs and 0.4 found in sea bass eggs (Emata et al., 2003). Unfortunately, their data were not derived from a rigorous design and ARA, specifically, was not the main subject of their study. However, their observations of higher ARA:EPA ratios in tropical species should be noted and considered in future work.

1.3.4. Vitamins

In addition to lipids, vitamins C and E have been found to be important in fish reproduction (partially reviewed by Emata et al., 2000). These authors reported that high levels of ascorbic acid have been found in fish oocytes and ovaries and concentrations of this chemical are known to change during the reproductive cycle of several fish species. Vitamin C has also been found to be important in vitellogenesis and in maintenance of sperm quality. Additionally, both vitamins C and E are powerful antioxidants and are capable of scavenging reactive oxygen species and regenerating additional antioxidants (Lee and Dabrowski, 2004).

Lee and Dabrowski (2004) reported that dietary vitamin C and E increased growth rates in yellow perch (*Perca flavescens*). They also demonstrated that dietary vitamin C improved semen quality, which led to increased fertilization and hatching rates. Vitamin C was found to be capable of regenerating vitamin E and vitamin E can spare vitamin C depending on tissue stores of each constituent. Emata et al. (2000) found that milkfish (*Chanos chanos*) broodstock diets supplemented with vitamin C alone, or in combination with vitamin E, produced eggs with higher viability, hatching rates and cumulative survival than broodstock fed the control diet.

The effects of vitamin E in fish reproduction have only recently been demonstrated. However, deficiency of vitamin E has been found to cause immature gonads in carp and reduced hatching rates in ayu (Izquierdo et al., 2001). Increased dietary vitamin E increased the percentage of buoyant eggs, hatch rates and percentage of normal larvae in red seabream. Additionally, deficiency of vitamin E resulted in low fertility and larval survival rates in gilthead seabream (Izquierdo et al., 2001). Vitamin E

is also capable of acting as an inter- and intra-cellular antioxidant, which enables homeostasis of metabolites in the cell and tissue plasma (Izquierdo et al., 2001). This characteristic may be of importance when feeding fish diets that contain high levels of HUFA, which are prone to oxidation. In this way, vitamin E can serve as an “*in vivo*” antioxidant helping to protect digested lipids within the cell from oxidation.

1.3.5. Carotenoids

Carotenoids are reported to be one of the most important pigment classes in fish, functioning in many physiological capacities such as protection from sunlight, serving as a pro-vitamin A source, and performing as antioxidants (Izquierdo et al., 2001). They have also been reported to be important for normal development of fish, although there is some controversy concerning their effects on egg quality in salmonids (Tacon, 1981). However, there is some recent experimental evidence to support the importance of carotenoids, particularly astaxanthin, in fish reproduction.

Vassallo-Agius et al. (2001) tested the effects of 10ppm astaxanthin in diets for striped jack (*Pseudocaranx dentex*) broodstock. Their results showed that egg quality characteristics, such as percent fertilization and percent hatch were not affected by astaxanthin at the level tested. However, egg production was significantly greater (nearly three-fold) in the fish fed the astaxanthin diet. Therefore, these authors concluded that astaxanthin was important for maturation and production of eggs, but not important in egg quality, per se, as this species does not incorporate astaxanthin into the egg.

In a follow-up study, Vassallo-Agius et al. (2002) tested the effects of a higher concentration of astaxanthin (30ppm) and the effects of paprika on spawning

performance of yellowtail (*Seriola quinqueadiata*) broodstock. These authors reported that, contrary to their results in previous studies with striped jack, astaxanthin supplemented diets positively affected egg quality in yellowtail. The objective of their current study was to determine if paprika addition would have similar effects. Their results indicated that paprika addition (2% by dry weight) significantly increased hatch rate and larval quality over the diet supplemented with astaxanthin. The authors concluded that the red and yellow xanthophylls present in paprika might be more bio-available than the pigments from the purified astaxanthin. They also acknowledged that other unknown factors originating from the paprika might also have been beneficial to the broodstock.

1.3.6. Nutrition and Coral Reef Fishes

Although much work has been carried out on marine food fish species, little has been reported for coral reef fish in captivity with regard to broodstock nutrition and its effect on egg quality and/or larval survival. However, there have been a few key field experiments that demonstrated the beneficial effects of enhanced parental nutrition on the eggs and larvae produced. McCormick (2003) reported the effects of feeding on coral spawn by damselfish (*Pomacentrus amboinensis*) on the eggs and larvae produced. In that study, female damselfish, which were found to be feeding on coral propagules produced during an annual spawning event, were compared to females that were not feeding on coral spawn. Coral propagules contain 50-70% lipid and are thus a very energy dense food source, especially when compared to that species' primary diet of

filamentous algae. It was predicted that the quality of eggs and larvae would be enhanced in fish that were feeding on this food resource.

The results indicated that, in just five days after the coral spawning event, fish that were feeding on the coral spawn had attained a higher condition factor and larger liver mass to body weight ratio than fish that were not feeding on the coral propagules. Also, eggs produced by the females that had fed on the coral spawn produced larvae with 25% larger yolk-sacs and 100% larger oil globules compared to larvae produced long after the spawning event and from two years prior.

To validate these observations, an additional feeding experiment was conducted, on the reef using the same species. The effects of supplementary feedings of high lipid food sources (pilchards and prawns) were tested by offering these items to selected pairs of fish for 5 minutes per day over a six-week period. Breeding pairs of damselfish were placed on isolated patch reefs and monitored for the duration of the experiment. Eggs and larvae from supplementary fed pairs were then compared to the pairs that were not supplementary fed.

Results from that experiment were similar to those obtained from the coral spawn data. Females that were supplementary fed attained a higher condition factor and greater relative liver weight. Additionally, larvae produced by these females exhibited significantly larger yolk-sac areas than larvae from the non-fed females. McCormick (2003) concluded that a pulse of high energy food, lasting for as little as 5 days, can have positive effects on larval quality, via increased yolk size, but the direct effects of varying yolk-sac size on larval survival have yet to be determined in tropical species.

1.4. Summary

The expansion of the marine ornamental aquaculture industry is in its infancy and has been limited by several “bottlenecks” including lack of suitable prey for first-feeding larvae and limited availability of larvae for feeding trials. Furthermore, the effects of broodstock condition on the eggs and larvae produced have been largely uninvestigated in marine ornamental species. In marine food-fish species, the progress of broodstock nutrition studies has been limited, in part, by the difficulty in providing sufficient number of replicates for experimental conditions, a complication that results from the fact that most commercially cultured marine finfish species are large, requiring large tanks and exceptional resources. In addition, many of those species take several years to reach sexual maturity, have a limited yearly spawning cycle of weeks to months, and are therefore very difficult and expensive to replicate under a controlled study. The use of limited replicates, and often long study durations, in this field of research has led to the slow growth of our knowledge and understanding of the effects of nutrition on egg production and egg quality.

Consistent production of high-quality eggs continues to be a significant bottleneck to the commercial culture of many marine fish, including ornamental species. Determining the effects of broodstock nutrition on egg production and egg quality continues to represent a significant challenge to fish culturists and is the focus of much study in the literature. Of particular interest, and perhaps of fundamental importance, are the effects of the n-3 and n-6 classes of the highly unsaturated fatty acids on egg quality. Over the past two decades, these lipids and their effects on egg quality and larval survival

have been widely investigated, but their effects, as they relate to broodstock contribution from diet to egg, are still widely debated and remain largely unstudied in many species.

Marine angelfish may serve as a valuable model for the continued expansion of marine ornamental aquaculture and could help to further our understanding of marine fish broodstock nutrition. These species produce eggs and larvae that share numerous characteristics with many other highly-valued ornamental species. Therefore, developing culture technologies for these species could facilitate the expansion of culture methods for many other reef species. While being representative of most marine ornamental species, with regard to type and size of eggs and larvae produced, angelfish of the genus *Centropyge* also exhibit several unique and desirable characteristics for studying the effects of parental diet on egg quality. These characteristics include, but are not limited to: 1) small size and ease of husbandry; 2) early onset of sexual maturity; 3) sexual dimorphism; 4) widespread availability via the marine aquarium industry; and, 5) year round (daily) production of eggs. In particular, it is this last characteristic that sets these fish apart and lends these species so well to the study of broodstock nutrition.

1.5. Current Research

The goals of this research were to do the following: 1) develop optimal husbandry practices for long-term maintenance of flame angelfish (*Centropyge loriculus*) spawning stocks, including development of a broodstock holding system, procurement of new stocks, developing quarantine and treatment procedures to limit pathogens and diseases, and conditioning of new pairs for spawning; 2) assess key environmental factors that may impact spawning performance of flame angelfish; 3) develop a comprehensive

baseline data set of flame angelfish spawning performance consisting of egg production and egg biochemistry from multiple spawning pairs; 4) formulate and test a diet to be used in multiple angelfish broodstock nutrition studies; 5) investigate the effects of broodstock diet on spawning performance and egg and larval quality in marine ornamental fish species, using flame angelfish as a model. In particular, the effects of the n-3 and n-6 highly unsaturated fatty acids (HUFA) such as DHA, EPA and ARA are discussed.

This dissertation is organized as a collection of papers, which have been arranged in the order in which the work was completed. Each chapter has been written as an individual paper, which will be submitted for publication in journals such as *Aquaculture*, and has therefore been formatted accordingly. These papers have not yet been submitted for publication as of the completion date of this dissertation.

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Chapter 2

DEVELOPMENT OF A MARINE ORNAMENTAL FISH BROODSTOCK LABORATORY FOR USE IN YEAR-ROUND AQUACULTURE RESEARCH

2.1. Abstract

The objective of this project was to develop a broodstock holding laboratory for assessing a variety of popular marine ornamental species' suitability to culture conditions in an indoor, recirculating system at The University of Maine. Simultaneously, this study sought to determine which species might be best suited to commercial culture in Maine utilizing indoor, recirculation systems. Upon completion of the laboratory, 48 pairs of marine ornamental fish broodstock from over 20 different species were evaluated for a period of one year. Pairs were monitored for frequency of spawning, quality of eggs (fertilization rate and hatching rate) and total number of spawns. Results indicated that most of the observed species initiated spawning after only a few months in the holding system and that the environmental parameters tested allowed for sustained spawning to occur across numerous species. Furthermore, dottybacks (*Pseudochromis sp.*) and pygmy angelfish (*Centropyge sp.*) proved easiest to condition, as these species exhibited the earliest onset of spawning and demonstrated the highest spawning frequency. These results demonstrated that marine ornamental species could be successfully maintained and conditioned to spawn under the conditions described. Therefore, the culture of marine ornamental species at The University of Maine is not only feasible, but could significantly enhance the current aquaculture research programs currently underway there.

2.2. Introduction

As reviewed in Chapter 1, the marine ornamental aquaculture industry is in its infancy and is faced with many challenges. Despite these challenges, some marine ornamental species (clownfish and dottybacks) have been commercially cultured and are now commonly available to hobbyists. While marine ornamental aquaculture may be a successful niche market unto itself, development of culture technologies for marine ornamental species could also aid the expansion of aquaculture of commercial food-fish species. Marine ornamental species may serve as valuable research models for marine finfish aquaculture, as most species spawn year-round, and in many cases, several times per month. This is in contrast to many commonly studied cold-water species such as cod or halibut that have brief, seasonal spawning seasons. Maintaining spawning populations of marine ornamental species would allow year-round research on marine finfish larviculture techniques that could then be applied to species that spawn less frequently. Therefore, one goal of this study was to create a marine ornamental fish broodstock laboratory at The University of Maine with which to pursue research addressing a number of these challenges.

The first objective of this dissertation research project was to design and construct a broodstock holding system capable of supporting consistent spawning across a wide range of marine ornamental species. Since research on tropical marine fish was previously unexplored at The University of Maine, it was necessary to determine which species would be best suited to the culture conditions in this environment. Once suitable species were identified, recommendations could be made as to which species might best

facilitate the expanded aquaculture research efforts at The University of Maine, and which species might lend themselves to a commercial operation in Maine.

2.3. Methods

2.3.1. System Design and Configuration

From September 2002 to May 2003, a new marine ornamental fish broodstock holding laboratory was designed and constructed at the Aquaculture Research Center (ARC) at The University of Maine in Orono, Maine. Design criteria incorporated provisions for both demersal and pelagic spawning tropical fish species, and included the use of various tank sizes to accommodate these species' range of spawning behaviors. The system (Fig. 2.1) was originally comprised of 75L glass aquaria (26), 160L glass aquaria (6) and 200L polyethylene tanks (8). The glass aquaria were used primarily for demersal spawning species, while the larger polyethylene tanks (Fig. 2.2) were used for pelagic spawning species, as those species typically require more room to accommodate their spawning rituals. All tanks received enough water flow to allow for a minimum of 8-12 tank exchanges per day. The glass aquaria were painted black on all the external surfaces except one to allow for easy viewing of the fish from the front, but which restricted the fish from seeing other pairs adjacent to them.



Figure 2.1. Photographs of the glass aquaria incorporated into the marine ornamental broodstock laboratory at the Aquaculture Research Center (ARC) at The University of Maine. The system consisted of 160L glass aquaria (left panel) and 75L glass aquaria (right panel). This portion of the system was used for demersal spawning marine ornamental broodstock pairs.



Figure 2.2. Photographs of the polyethylene tanks incorporated into the marine ornamental broodstock laboratory at the ARC. Tanks were originally configured with remote egg concentrators (left panel). After redesigning the egg collectors, the tanks were arranged in a new configuration to allow more replicates (right panel). This portion of the system was used for pelagic spawning marine ornamental broodstock pairs.

All of the tanks were incorporated into a single recirculating aquaculture system (RAS) and shared a central filtration system (Fig.2.3) consisting of a Kaldness™ “moving-bed” biological filter, two-stage (100 micron and 10 micron) mechanical filter,

protein skimmer and ultraviolet sterilizer. Additionally, shredded PVC was placed inside the aerated head tank to serve as surface area for nitrifying bacteria to provide additional biological filtration of the water prior to return to the fish holding tanks. Design, selection and engineering of the systems' filtration components followed recommendations by Timmons et al. (2001) and Escobal (1996). Briefly, the main purposes of the RAS were to allow re-use of the system water by: 1) elimination of nitrogenous waste (Total ammonia-nitrogen (TAN) $\text{NH}_3\text{-N} + \text{NH}_4\text{-N}$ and Nitrite, NO_2) via the biological filter; 2) removal of suspended solids through mechanical filtration; 3) removal of dissolved organic compounds via foam fractionation; and 4) reduction of the occurrence of pathogenic bacteria, viruses and parasites by exposure to UV radiation. Further information on each of the systems' components can be located in excellent overview of recirculating aquaculture systems by Timmons et al. (2001).

The main filtration area was intentionally located away from (downstairs and underneath) the broodstock holding area to reduce noise and vibrations associated with the pumps and filters. Saltwater for the system was created using a commercially available marine salt mix (Crystal Sea™ Marine Mix) mixed with reverse osmosis filtered water. Approximately 10% of the system volume was exchanged weekly with new artificial saltwater.

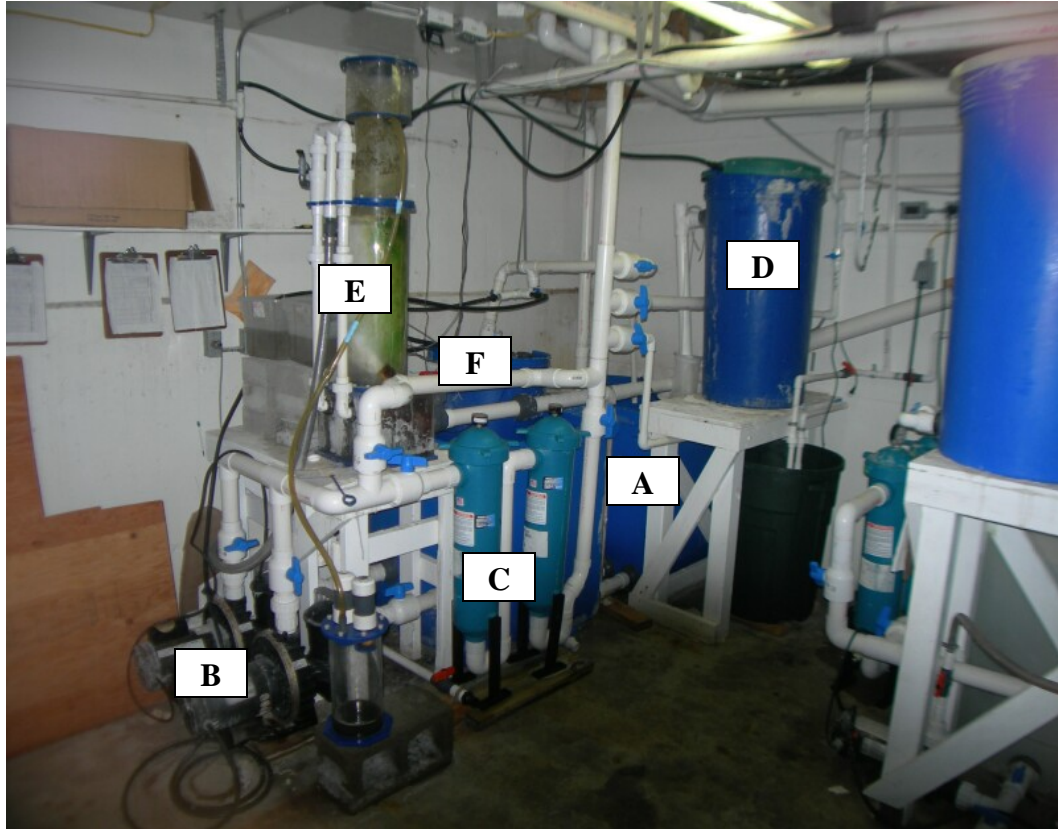


Figure 2.3. Photograph of the filtration equipment for the marine ornamental broodstock system at the ARC. Water leaving the tanks in Figs. 2.1 and 2.2 terminated into the main sump (A). From there, water was pumped (B) through the two-stage mechanical filter (C). Water directed through (C) was split to go to either the moving-bed biological filter (D), to barrels containing submerged heaters (F) or directly back to the head tank in the broodstock lab (not pictured). A separate pump directed water from the sump to the protein skimmer (E), which discharged into the sump (A). The pictured components were located in a separate room to minimize noise and disturbance for the broodstock pairs.

Lighting for the system was provided by very high output (VHO) fluorescent bulbs that were selected for their ability to approximate natural sunlight. These lights were controlled by computerized dimmers, which simulated daily dawn and dusk cycles while controlling the photoperiod. The photoperiod was fixed on a constant, long day-length (14hL:10hD) to simulate summer conditions year-round (for northern hemisphere

tropics and subtropics). The temperature of the system was maintained at $27\pm1^{\circ}\text{C}$ by submersible heaters in the sump and an air conditioning unit capable of heating and cooling the air within the lab. The salinity of the system was maintained at 32ppt by utilizing a freshwater dosing pump controlled by a computerized controller (AquadyneTM-Octopus). This same controller also continuously monitored the system temperature, salinity, pH and dissolved oxygen. If any of the system water quality variables were out of range, the controller would call a pager alerting a staff person. A schematic diagram of the system is presented in Figure 2.4.

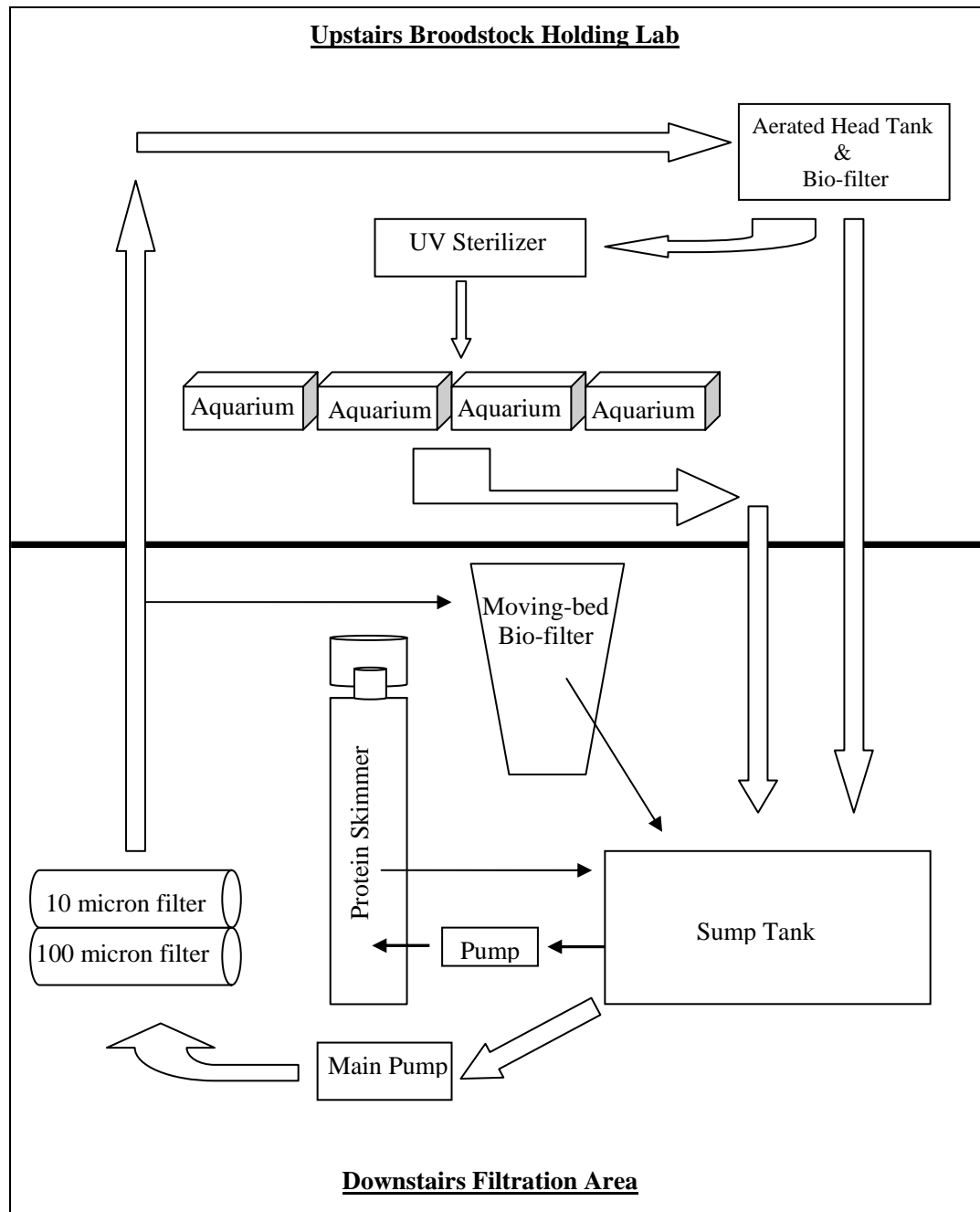


Figure 2.4. Schematic diagram of marine ornamental broodstock holding system at the ARC. Block arrows indicate main water flow pattern. Line arrows indicate side-loop water flow through moving-bed biological filter and protein skimmer.

2.3.2. Acquisition of Marine Ornamental Fish Broodstock

2.3.2.1. Demersal Species. In June of 2003, we procured a number of pairs of different demersal spawning species (primarily clownfish and dottyback species) to test the newly constructed system's ability to recreate conditions necessary for year-round reproductive output by tropical marine species. We initially selected clownfish and dottybacks as both species had been spawned and commercially reared in recirculating systems (Callan, unpub. data). Moreover, we wanted to ensure that our system design was sufficient to replicate spawning in these species prior to moving on to species that were potentially more challenging, such as angelfish. Wherever possible, established breeding pairs were preferentially selected to minimize the conditioning time required to obtain spawning. Initial pairs were selected from two commercial suppliers (Inland Aquatics Inc., Terre Haute, IN; Mangrove Tropicals Inc., Kahuku, HI.) and shipped to The University of Maine between the months of June and August 2003.

Upon arrival, new pairs were placed into isolated 75L or 160L glass aquaria, which served as quarantine tanks for 8 weeks. The tanks were kept bare, with the exception of some PVC pipes or clay pots, which were provided as hiding and spawning areas for the fish. The tanks were individually filtered using pre-conditioned airlift sponge filters and 25% of the tank water was exchanged weekly. The fish were monitored closely, during the quarantine period, for any signs of bacterial or parasite-related diseases, which were treated if necessary. The fish were offered a wide variety of commercial aquarium diets and were typically fed two to three times daily. The quarantine tanks were siphoned daily to remove any uneaten food and any solid wastes. Following the two-month quarantine period, each pair of fish was given a 2-4 minute

freshwater dip to exclude any external parasites prior to being transferred to the main broodstock holding system.

2.3.2.2. Pelagic Species. Flame angelfish were among the first pelagic spawning species to be acquired and were first obtained from a commercial aquarium fish supplier (The Marine Center, www.themarinecenter.com) in January 2004. Twelve individual fish were purchased (established pairs were unavailable) and were paired on arrival. After acclimation, a larger individual (presumed to be male) was paired with a smaller individual (presumed to be female) to form the “pairs” (Fig. 2.5).

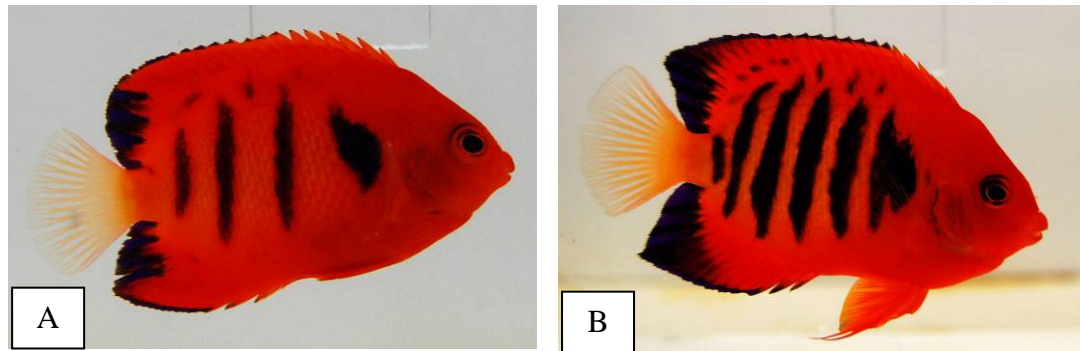


Figure 2.5. Photographs of male and female flame angelfish. (A) Female flame angelfish are characterized by their smaller size, less than 4 vertical stripes, more squared off posterior ventral and dorsal fins, and less prominent blue and black coloration of posterior. (B) Male flame angelfish are characterized by their larger size, more than 4 vertical stripes, more pointed posterior ventral and dorsal fins, and striking blue and black coloration of posterior.

When pairing the fish, the number of stripes on each fish was counted. Whenever possible, a larger individual with five or more stripes per side was paired with a smaller individual with four or fewer stripes per side. Five pairs were established based on these external characteristics; the pairs were then placed into isolated, 160L glass aquaria (quarantine system described earlier) for a period of two months. The aquaria were blacked out on all sides except one, which allowed for easy observation of the fish.

Large, 3 and 4-inch diameter PVC-pipe fittings were placed in the aquaria to serve as hiding places for the fish. The fish were visually monitored closely for the first 3 days to ensure no aggressive behavior was occurring between the newly formed pairs. The fish were fed a varied diet, consisting of dry and frozen commercial aquarium foods, four times daily. The tanks were siphoned daily to remove any uneaten food or feces and water quality parameters were monitored daily. All five pairings were successful, as no aggressive behavior was observed and within days of arrival the fish seemed well acclimated to our lab conditions. No treatment for disease or infection was necessary during the entire quarantine period.

2.3.3. Broodstock Conditioning

2.3.3.1. Clownfish, Dottybacks and Other Demersal Species. Following the quarantine period, demersal spawning species including clownfish and dottybacks were placed into 75L or 160L glass aquaria in the main broodstock holding system. Each tank held one pair of fish and was kept free of decoration except for either a clay flowerpot or PVC pipe “den”. Clownfish tanks contained one clay flowerpot, which served as a hiding place and spawning surface. The smooth surface of the clay flowerpot proved to be an ideal spawning site for clownfish and provided an easy way to remove the adhesive eggs from the tank prior to hatching. A photograph of clownfish tending their eggs on their pot can be seen in Figure 2.6. Clownfish proved relatively easy to condition, and pairs spawned when provided with warm water temperature (27-28°C) and long day length (14hL: 10hD). Clownfish readily accepted a variety of commercial aquarium diets, although spawning was improved (increased spawning frequency, higher fecundity and

greater number of hatched larvae produced) when their diet was supplemented with a mixture of raw fish, squid and scallops. Generally, the fish were fed four times per day and the types of food items offered varied from day to day.



Figure 2.6. Photograph of Common clownfish (*Amphrion ocellaris*) tending to their eggs on the side of a flowerpot. Photograph by Larry Ayotte for The University of Maine.

Dottybacks and gobies were provided with PVC “dens” in which they spent the majority of their time and also laid their eggs. The PVC dens were constructed by capping off a 6-inch section of 2 inch PVC pipe on each side. A small (<1 inch) hole was then drilled into one of the caps, allowing access to the den from only one side. A photograph of this constructed den can be seen in Figure 2.7. Dottybacks accepted a variety of commercial aquarium diets, though spawning was improved (increased spawning frequency, higher fecundity and greater number of hatched larvae produced) when their diet was supplemented with frozen mysis shrimp as well as a mixture of raw fish, squid and scallops. Generally, the fish were fed four times per day and the types of food items offered varied from day to day. The system’s water temperature (27-28°C) and increased day length (14hL: 10hD) also promoted spawning in these species.



Figure 2.7. Photograph of an Orchid dottyback (*Pseudochromis fridmani*) pair and their PVC “den.” Photograph by Larry Ayotte for The University of Maine.

2.3.2.2. Angelfish and Other Pelagic Spawning Species. After the 8-week quarantine period, the pairs of angelfish were moved to the broodstock holding system. Each pair was placed in a specially designed 200-liter tank (Fig. 2.8). The tanks were bare except for a few pieces of PVC fittings, which served as shelters for the fish. These tanks were configured as follows to accommodate the angelfish’s pelagic spawning behavior. Each tank was equipped with a bottom and surface drain; the surface drain was used from dusk till dawn to collect any eggs produced in the tank. Each tank’s surface drain allowed water to pass through an individual egg collector, which was checked nightly. The first eggs were collected in March 2004, approximately 1 month after the pairs were introduced into the broodstock system and only 3 months after pairing the newly acquired fish. As with the other broodstock species, the angelfish were fed four times per day and the types of food items offered varied from day to day. Their diet included commercial aquarium diets supplemented with frozen mysis shrimp as well as a

mixture of raw fish and squid. The system's water temperature (27-28°C) and increased day length (14hL:10hD) also promoted spawning in these species.



Figure 2.8. Photograph of the pelagic tank configuration used for spawning flame angelfish at the ARC.

2.4. Results

2.4.1. Clownfish and Dottyback Spawning Behavior

Following a brief (20-30 days) period to allow acclimation to our system, several (5) pairs of clownfish and dottybacks began to spawn. Observed spawning behavior of clownfish in our system closely resembled that reported by Hoff (1996) and Wilkerson (2001). Clownfish utilized the flowerpots as a substrate upon which to lay their adhesive

eggs. Female clownfish, typically the largest of the pair, would exhibit noticeable abdominal swelling as spawning became imminent. Additionally, the male clownfish would aggressively begin to “clean” the spawning surface of the flowerpot by scraping it with his teeth. This cleaning activity would typically begin on the day prior to spawning, but would intensify on the day of spawning. The final indicator that spawning was about to occur was the appearance of the female’s ovipositor, which was normally not visible. Clownfish pairs typically spawned in the late afternoon or early evening prior to the lights going out. However, we could not discern a clear pattern associated with these spawning events, and occasionally pairs would spawn at unpredictable times.

Clownfish generally produced 500 to 1000 eggs per spawn. These eggs were usually laid in a tight, round to oval, cluster pattern, originating from the center of the cluster outward. Occasionally the eggs would be loosely scattered around the spawning site, indicating that the pairs’ spawning behaviors were a little out of synchrony. Loosely scattered eggs were also indicative of poor quality eggs, which generally did not complete development. To lay the eggs, the female would make a slow moving pass over the spawning area, depositing eggs a few at a time. The male would then follow closely behind her, fertilizing the eggs. Normally, the entire spawning event would take less than 1 hour to complete. The normal embryonic development time for clownfish eggs was from 8-9 days. During this development, the male would typically “care” for the eggs by fanning them with his pectoral fins and removing any dead eggs or debris from the egg mass with his mouth. Eggs would normally hatch on the evening of the 9th day, shortly after the lights went off. The time between spawning events varied, but typically ranged from 11-14 days, resulting in approximately two spawning cycles per month.

Dottyback spawning behavior was nearly identical to that reported by Moe (1997) with individual fish exhibiting noticeable signs that alerted us to potential spawning activity. As in clownfish, the female (the smaller of the pair) would exhibit noticeable abdominal swelling as spawning became imminent. Additionally, the male would begin to repeatedly attempt to “coax” the female into his den. This behavior was typically observed only on the day of spawning and consisted of the male hovering near the entrance to his den, posturing his body in displays for the female. The male would repeatedly go in and out of the den in attempt to get the female to follow. Eventually, the female would enter the den and spawning would commence.

Dottybacks routinely produced 1000-2000 eggs per spawn; the eggs were laid in a single strand and held together by sticky, adhesive threads. As the eggs were laid by the female, the male fertilized them as he wrapped himself around the female in the den. The male and female continued to wrap themselves around the eggs as they were laid and fertilized, creating a spherical-shaped egg mass. Once the spawning concluded, the female quickly left the den. The male then stayed inside the den, guarding the egg mass and periodically “shuffling” it around in the den.

The normal embryonic development time for dottyback eggs was from 5-6 days. During this development, the males would typically “care” for the eggs by fanning them with his pectoral fins, shuffling the egg mass around in the den, and removing any dead eggs or debris from the egg mass with his mouth. Eggs would normally hatch on the evening of the 6th day, shortly after the lights went off. The time between spawning events varied, but typically ranged from 7-10 days, resulting in three to four spawning cycles per month.

2.4.2. Angelfish Spawning Behavior

Spawning behavior was more difficult to observe in the opaque, polyethylene tanks and could only be viewed from above. However, when observed, the flame angelfish spawning behavior closely matched that described by Bauer and Bauer (1981). The angelfish pairs spawned prior to, or just after dusk, which was set for 1700 as controlled by our automatic dimmers. Eggs were first observed in the collectors from 1615 to 1730. If no eggs were observed by 1800, the pair did not spawn at all that evening. Four out of five total pairs produced eggs. Periodic inspection of the egg collectors over a 2-week period revealed that three of the five pairs consistently produced 250-500 eggs per night. The remaining pair produced <100 eggs per night. Egg collectors were then sampled nightly from May through early June 2004. During that period, three of the pairs produced (~500) eggs each night. The remaining pair produced eggs (<100/night) sporadically over that period, with no observable pattern. Eggs were considered fertile if cell division was taking place when the eggs were collected (a few hours post-spawning). Fertilization rates were determined by collecting a sub sample of approximately 100 eggs and counting the number of developing embryos divided by the total number of eggs. Fertilization rates were normally over 75% for the three consistently spawning pairs and fluctuated from 25-80% for the sporadic spawning pair. Eggs collected and incubated from all angelfish pairs had very poor hatch rates (<30%) and survival of the newly hatched larvae was very poor. No surviving larvae remained to sample following 2 days post-hatch.

2.4.3. Egg Collector Modification

Observed high egg mortality of the pelagic angelfish eggs could have been an artifact of the initial egg collector design. In the initial design, eggs were skimmed from the surface of the tanks and traveled down a drainpipe that terminated into a submerged mesh net. The turbulence and splashing of water into these mesh “concentrators” may have been too hard on the developing eggs. Alternate collectors were designed and modeled after those at the Oceanic Institute in Hawaii (C. Laidley, personal communication.). This new design minimized the distance the eggs traveled to the concentrator and greatly reduced turbulence within the concentrator. Varied egg collector configurations were tested nightly from 6/8/04 to 6/25/04. Diagrams of the original tank configuration and the finalized version of the collectors are presented in Figure 2.9. By reducing the water turbulence in the egg collector egg viability increased noticeably. Eggs could be left in the collectors until the following morning (instead of collected the night of spawning) and then transferred to a hatching tank. Utilizing this modified collector (Figs. 2.10 and 2.11) increased hatch rates to nearly 100%.

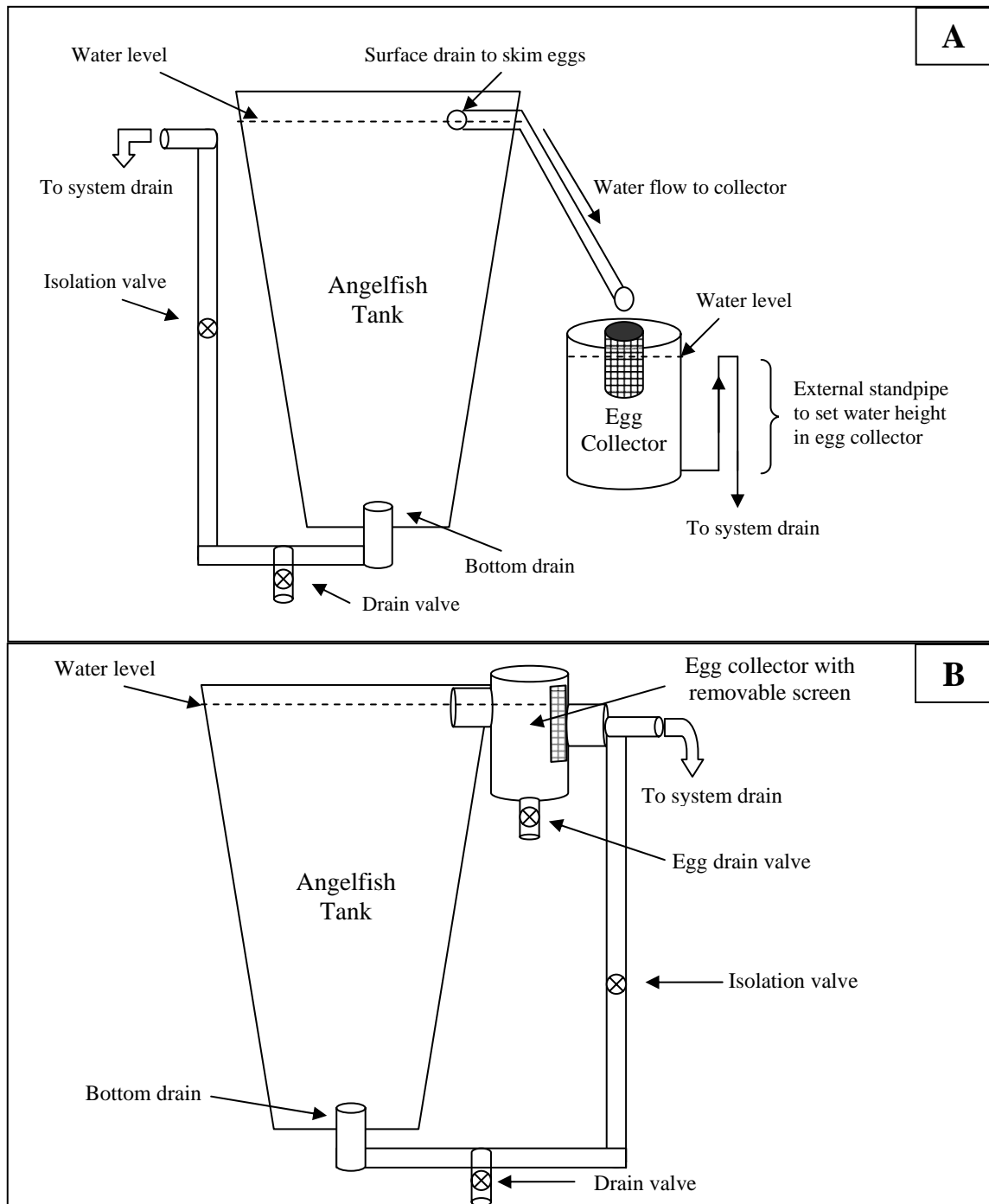


Figure 2.9. Schematic diagrams of angelfish holding tanks and egg collectors at the ARC. (A) Original angelfish tank and egg collector configuration where eggs would be collected in a 200 micron mesh net submerged in a remote tank. (B) Modified egg collector design where eggs were concentrated in a PVC tube adjacent to the tank. This design minimized turbulence as there were no drops or bends of plumbing and was much gentler on the eggs.



Figure 2.10. Photograph of modified egg collector used for concentrating flame angelfish eggs. Removable screen would be slid into place at the end of the day. Eggs would be concentrated in the main body of the collector and then could be drained out in the morning.



Figure 2.11. Top view of modified egg collector showing very little disturbance of water in the collector, despite high flow-rates through the unit. The design allowed for the eggs to travel completely submerged into the collector and remain nearly undisturbed at the water surface for the duration of the evening.

After maintaining the selected marine ornamental species for approximately one year, we reviewed the spawning data we had compiled. The evaluation criteria we used to determine if a species were suitable for further study at The University of Maine were based on: 1) time to first spawn; 2) consistency of spawning; 3) hatchability of eggs and, 4) quality of eggs and larvae. A total of 48 pairs from 21 species were evaluated. A summary of our spawning data for the observation period from June 2003 to June 2004 is presented in Table 2.1.

Table 2.1. Spawning data from observed marine ornamental species (6/1/2003-6/30/2004). Division line separates demersal spawning species (top) from pelagic spawning species (bottom)

Scientific name	Common Name	Number of Pairs Evaluated	Number of Pairs that Spawned	Mean Time to 1 st Spawn (months)	Total Spawns
<i>Amphiprion ocellaris</i>	Common Clownfish	6	3	6	30
<i>Amphiprion percula</i>	Percula Clownfish	1	1	1	25
<i>Amphiprion frenatus</i>	Tomato Clownfish	2	1	1	18
<i>Amphiprion clarkii</i>	Clark's Clownfish	1	0		0
<i>Amphiprion melanopus</i>	Melanopus Clownfish	1	1	2	3
<i>Pseudochromis fridmani</i>	Orchid Dottyback	6	6	2	112
<i>Pseudochromis aldabraensis</i>	Neon Dottyback	5	3	4	20
<i>Pseudochromis springeri</i>	Springeri Dottyback	3	0		0
<i>Pseudochromis flavivertex</i>	Sunrise Dottyback	1	1	1	1
<i>Pseudochromis spendens</i>	Splendid Dottyback	3	1	2	15
<i>Elacatinus oceanops</i>	Neon goby	2	1	8	3
<i>Gobiodon okinawae</i>	Clown goby	2	1	4	15
<i>Gobisoma macrodon</i>	Tiger goby	1	1	1	8
<i>Pterapogon kauderni</i>	Bangaii Cardinal	1	1	3	2
<i>Nemateleotris magnifica</i>	Firefish	2	0		0
<i>Liopropoma rubre</i>	Swissguard Basslet	1	1	4	4
<i>Pseudanthias squamipinnis</i>	Lyretail Anthias	1	1	2	10
<i>Paracheilinus sp</i>	Flasher wrasse	1	0		0
<i>Centropyge loriculus**</i>	Flame Angelfish	6	4	3	25
<i>Centropyge interruptus</i>	Japanese Angelfish	1	0		0
<i>Synchiropus splendidus</i>	Mandarinfish	1	1	1	2
Total	21	48	28		296

** Flame angelfish only observed from 4/04 through 6/04

Amphiprion ocellaris clownfish took much longer to acclimate to spawning condition than many of the other selected species. Although two previously spawning pairs of *Amphiprion ocellaris* were evaluated, only one pair spawned within a month of acclimation to the system. The other pair took 9 months before spawning. Four adult pairs of *Amphiprion ocellaris*, which were not yet spawning, were also evaluated. Although, of these four pairs, only one started spawning 12 months after they were acquired. The other species of clownfish responded sooner, with many pairs spawning only a few months after acclimation. A similar pattern was seen with the dottybacks and the other selected species. Of the pairs that did spawn, many spawned within one month of conditioning and almost all pairs were spawning within the first 3 months. Of the 48 pairs evaluated, 28 produced eggs during the project period.

Once spawning initiated, consistency among most pairs was very good. Two spawns per month were routinely recorded for many of the clownfish species and as many as four spawns per month for the dottyback species. For a few of the pelagic spawning species, daily spawning over protracted time periods was observed. This frequency is similar to observations reported by Bauer and Bauer (1981) for these families. It is worth noting that captive spawning in *Pseudanthias* has not been reported previously.

The hatching success of all the eggs produced was very high. In most cases with the demersal eggs, 100% fertilization and hatch rates were observed. This was to be expected among species that utilize this spawning strategy, as the adults remain very close to their eggs and protect them throughout development. Additionally, fertilization

and hatch rates among the pelagic eggs that were produced by the angelfish, lyre tail anthias, and mandarinfish were generally very high (>75%).

2.5. Discussion

This project demonstrated the successful design and construction of a recirculating broodstock system that supported spawning across a wide variety of marine ornamental fish species. The system was configured using readily available components allowing its design to be replicated across many scales. The system proved flexible in that tanks could be added or removed from the system with minimal impact to the remaining tanks, and the environmental controls allowed for endless possibilities for photoperiod and temperature manipulation. Constant “summer” temperature and photoperiod proved to be effective for conditioning most of the species evaluated, as nearly all the species spawned year-round under those conditions. It is possible that by cycling temperature and photoperiod, more seasonal spawning patterns might have been realized. However, most species were quite adaptable to the constant conditions provided.

The observed rapid conditioning could be attributed to careful acquisition of mature fish. By selecting and obtaining previously spawning pairs (when possible), conditioning time for most species was significantly reduced, as spawns from many species were attained within the first few months of conditioning. It is probable that this rapid conditioning is atypical for a few of the species evaluated, such as clownfish, as some of the pairs of *Amphirion ocellaris* took over a year to initiate spawning. Additionally, some of the pairs that were evaluated never spawned, indicating that they were either not responding to the conditions provided, or that they were not yet mature.

However, species such as dottybacks and angelfish conditioned relatively quickly, even if not acquired as established pairs. Despite the fact that it was often necessary to establish newly formed pairs of these species, observed spawning in these species was routinely observed in less than 4 months after acquisition.

Based on these results, it is recommended that dottybacks and angelfish be considered for future research at The Aquaculture Research Center. Results of this project have shown that these species take only a few months of conditioning prior to spawning. Once conditioned, the pairs will produce many eggs, very frequently. Additionally, both species will spawn year-round under the conditions described in this study. Furthermore, dottybacks produce relatively large larvae, which can be reared on rotifers and *Artemia sp.* These characteristics make them amenable as models for species such as cod, which exhibit similar larval developmental traits (Callan, 2000). Angelfish can spawn daily and therefore may serve as ideal models for testing effects of various treatments on egg production and egg quality. While their larvae are very small and have not been successfully cultured using rotifers, techniques developed to further their culture may be applicable to other difficult-to-rear coldwater species such as haddock and halibut.

Although not part of the original evaluation project, six species of clownfish and dottybacks were successfully reared through the juvenile stages to saleable size in the recirculating culture systems at the ARC in Maine. Furthermore, spawning pairs of dottybacks were obtained from the first generation (F1) of production. These achievements indicate that the culture of marine ornamental species at The University of Maine is not only feasible, but could significantly enhance the current aquaculture

research programs currently underway there. Utilizing these species might better facilitate the year-round use of facilities and resources on campus while providing additional, unique and exciting opportunities for students. The current rising interest in coral reef ecology and marine ornamental aquaculture could be leveraged to provide an additional and dynamic component to The University of Maine's existing marine biology and aquaculture degree programs.

2.6. Acknowledgments

We wish to thank Neil Greenberg for assistance with facilities construction and Jacque Hunter for assistance with live food production and daily technical needs at the ARC. Thank you also to the many undergraduate students who assisted us with this project; Natasha, Gordon, Heather, Matt, Davin, Steve, Mahima, Troy and Sarah, your assistance in helping to care for the fish and systems made this project possible. Marine ornamental broodstock were provided by Sea & Reef Aquaculture, LLC through a grant from the Maine Technology Institute. Funding for the development and maintenance of the broodstock laboratory was provided by The University of Maine, School of Marine Sciences.

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Chapter 3

EFFECTS OF COPPER EXPOSURE ON MARINE ORNAMENTAL FISH REPRODUCTION AND SURVIVAL

3.1. Abstract

This study was carried out to determine if exposure to copper would cause mortality in flame angelfish (*Centropyge lorciulus*) and affect reproduction of the orchid dottyback (*Pseudochromis fridmani*). Flame angelfish were exposed to copper in a series of toxicity assays. In the first assay, angelfish were exposed to copper at 0.00, 0.05, 0.10, 0.15, 0.20 and 0.25mg/L for a period of 48 hours (n=5). In the second assay, angelfish were exposed to copper at 0.00, 0.10, 0.15 and 0.20mg/L for 196 hours (n=8). In a third experiment, orchid dottyback broodstock pairs (n=3) were maintained and monitored for reproductive performance (spawn frequency, fecundity, fertilization rate and survival of hatched larvae) while in copper-free water (0.00mg/L) or water treated with copper (0.10mg/L). Results of the toxicity assays revealed that flame angelfish were acutely sensitive to copper in the first trial, where 60% of the fish exposed to the 0.25mg/L level died within the first 12 hours of exposure. Likewise, flame angelfish exposed to 0.15 and 0.20mg/L exhibited 40% mortality. In the second assay, flame angelfish also exhibited increased mortality (25%) at the highest level tested (0.20mg/L), though the onset of mortality, in that experiment, was delayed until after 120 hours of exposure. Results of the third experiment demonstrated that copper exposure at 0.10mg/L significantly reduced fecundity and negatively affected embryonic development from orchid dottyback broodstock. However, upon replacement into copper-free water, subsequent fecundity, embryonic development and larval survival characteristics were not significantly

different from their pre-exposure values. The results of these combined experiments indicated that elevated copper levels can cause acute mortality in flame angelfish and significantly reduce the reproductive performance of orchid dottyback broodstock. Therefore, the use of copper as a therapy for external parasites in these species is cautioned.

3.2. Introduction

During the commissioning of a new marine ornamental fish broodstock laboratory at The University of Maine, we experienced an episode of acute mortality of nearly all our flame angelfish broodstock in response to what appeared to be an unidentified toxin in the system. Abruptly one day in June 2004, our flame angelfish were observed to be swimming erratically at the surface of their tanks and some appeared to be “gasping.” Closer examination of all our angelfish revealed that most were exhibiting rapid respiration and some appeared listless. The angelfish were promptly removed from the system and placed into new tanks in another system. Unfortunately, most of the fish succumbed to the apparent toxin prior to being moved. Those fish that had survived the move, recovered rapidly once in new water. The remaining fish in the system (anemonefish and dottybacks) appeared normal and otherwise unaffected; however, their spawning performance and egg quality did significantly decline for a prolonged period following this event. After more than two months of investigation, we concluded that the cause of the mortality was likely an elevated copper level in the system caused by the accidental dropping of copper wire into one of the sumps in our recirculation system.

About two weeks prior to the incident, an electrical junction box was installed in an area near our main filtration area. Upon inspection of our systems, we found pieces of copper wire insulation and small clippings of mostly dissolved copper wire in the system sump, near the main pump intake. It is likely that these clippings were byproducts of the recent electrical work, and were accidentally dropped into the system. Months after the copper wire was found and removed, the system continued to exhibit an elevated copper level of 0.10 to 0.20mg/L, which is nearly the therapeutic level for treatment of marine fish parasites (Noga, 2000). Since copper was never utilized as a therapy in this system, it was not suspected as a possible toxin and the actual copper level at the time of the fish mortalities was not recorded. Therefore, it is possible that the copper level at the time of the mortality was considerably higher, as we had made several large (>50%) water changes to the system immediately following this event, and prior to measuring the dissolved copper levels. What remained unclear is why only the angelfish exhibited this acute response, while the other species in our system appeared mostly unaffected.

Copper is an essential element required by all living organisms, but it can be toxic to aquatic species when present at elevated concentrations (Grossel et al., 2003, 2004a, 2004b; Handy, 2003). For example, copper is widely used as an algacide and molluscicide (Paris-Palacios et al., 2000) and frequently employed as a treatment for pathogenic parasites of fish (Bassleer, 1996; Noga, 2000; Perschbacher, 2005). Although toxicity arising from dietary exposure to copper generally only occurs at very high levels, exposure to low levels of dissolved copper in the holding water can cause toxic effects in aquatic organisms (Grossel et al., 2004a).

While the mechanisms of copper toxicity of freshwater fish have been studied in some detail, much less is known about the mechanisms of copper toxicity of marine teleost fish (Grossel et al., 2003). It has been suggested that marine fish are generally less sensitive to water borne copper exposure than freshwater fish (Grossel et al., 2003). Calcium and sodium in the water have been found to reduce both copper uptake and toxicity in freshwater fish and it is suggested that the high concentrations of these ions in seawater help to reduce the acute sensitivity of marine teleosts to copper (Grossel et al., 2003). Increasing water hardness, addition of organic substances, and increasing pH will also reduce the toxicity of copper in fresh water (Handy, 2003).

The physiological mechanism of acute copper toxicity to fish is well known (reviewed in Taylor et al., 1996). Acute copper toxicity in fish is caused by direct target effects on the gill epithelium (Noga, 2000). Acute copper exposure causes gill edema and epithelial lifting (Handy, 2003). Edema of the gill is followed by accumulation of solutes in the epithelial cells and an influx of water into the cells, leading to loss of ionoregulatory control. Efflux of electrolytes from the blood over the gill epithelium then results in cardiovascular failure and death (Handy, 2003).

While the acute effects of copper toxicity are well studied, the effects of chronic copper toxicity are not well documented (Handy, 2003). However, it has been shown that chronic (4 weeks or more) copper exposure can result in altered bodily functions and physiological changes across a range of body systems. The physiological processes that have been found to be affected by chronic copper exposure include altered cell type and turnover in gut epithelium, changes in ionoregulatory physiology, altered immunity, change of swimming speeds to preserve metabolic scope for aerobic metabolism, and

altered reproductive strategy (Handy, 2003). Handy (2003) described these physiological responses to chronic copper exposure to be caused by Cu-dependent stimulation of generic stress responses or the interference of copper on specific neuro-endocrine processes that normally require copper to operate. Handy (2003) suggested that the endocrine disrupting effects of chronic copper exposure and the effects of copper on body systems are likely the opposite sides of the same toxicological process.

The objectives of the present study were to: 1) determine if exposure to copper would affect the survival of a commonly traded marine ornamental fish, the flame angelfish and 2) determine if exposure to copper would affect the reproductive performance and embryonic development of a cultured marine ornamental fish, the orchid dottyback. Due to the ubiquitous use of copper as a treatment for external parasites in the marine aquarium industry and the documented acute and chronic effects of copper on fish, coupled with our unexpected episode with the flame angelfish, we considered it necessary to investigate possible impacts such treatments may have. Our goal was to determine if the level of copper that we recorded after the mortality event could have caused the observed mortality and contributed to the decline in reproductive performance of our other pairs of fish.

3.3. Methods

3.3.1. Experiments 1 and 2: Effects of Copper Exposure On Survival of the Flame Angelfish (*Centropyge lorciulus*)

3.3.1.1. Experiment 1. The first of a series of copper toxicity assays was a 48-hour trial completed in June of 2005, at the Oceanic Institute (OI) in Hawaii. This first copper toxicity trial investigated the effects of copper exposure on flame angelfish (*Centropyge lorciulus*) at 0.0, 0.05, 0.10, 0.15, 0.20, and 0.25mg/L. Five replicate fish were tested in each treatment except the control, which contained three replicate fish. This assay was done in 30 identical (75L) tanks (Fig. 3.1), with one fish stocked per tank. The majority of fish that comprised each treatment group had been at OI for several weeks prior to the start of the trial and were well acclimated to the aquarium conditions. Approximately two new fish per treatment group were added the day prior to the experiment to increase the number of replicates. Each tank had a separate external filter and light aeration was provided via an air stone in each tank. The tanks were exposed to ambient photoperiod (filtered natural sunlight) and the temperature was maintained between 24-27°C. The salinity of the water was approximately 32ppt. New seawater was provided to each tank at a rate of ~25ml/minute (50% exchange per day). Copper was dosed directly into the tanks as copper sulfate using a commercially available preparation (Red Sea “Paracure™”) and the total copper level was determined by colorimetric analysis (Bicinchoninate method; Hach #2504025) twice daily at 0900 and 1500 for each tank using a Hach™ DR/890 colorimeter. When necessary, copper was re-dosed to maintain the desired treatment level. Mortality was monitored hourly for the first 12 hours, followed by every 12 hours for the duration of the experiment.



Figure 3.1. Photograph of the bioassay system at the Oceanic Institute. Each 75L tank was equipped with separate lighting and filtration and could be run as entirely flow-through, re-circ, or a combination of both.

3.3.1.2. Experiment 2. The second copper toxicity assay was completed in January of 2006, using the bioassay system (Fig. 3.1) at OI, but with a modified configuration. The same 75L tanks were utilized, but they were re-plumbed to allow for continuous exchange of natural seawater (6 tank exchanges/day). Temperature was maintained at 26-27°C and salinity was maintained at 32ppt. Additionally, new lighting and filtration were added to each tank in the form of an Eclipse™ hood (Marineland Aquarium Products, Inc.). The lighting system was set on a timer to allow for 14 hours light and 10 hours dark. Four treatment levels (0.0, 0.10, 0.15 and 0.20mg/L copper) were tested, with one fish stocked per tank (n=8). For this experiment, all fish were stocked at the same time (24h prior to start of trial) and originated from a local marine fish importer (Hawaiian Sealife Inc, Honolulu, HI).

Copper was added to the incoming seawater as copper sulfate using Dosatron™ (DI 1500) proportional mixing pumps, which were plumbed into the incoming seawater lines (1 dosing unit per treatment). A 100mg/L copper solution was made daily using distilled water and cupric sulfate pentahydrate (Fisher Scientific). This stock solution was dosed into the incoming seawater lines for the respective treatment groups to maintain the desired treatment levels for the duration of the study. This configuration allowed for very tight control over the copper levels tested while maintaining excellent water quality in the aquariums. Total copper levels were tested once daily for each treatment (n=3 samples/treatment) using colorimetric analysis as described for Experiment 1.

3.3.2. Experiment 3: Effects of Copper Exposure on Reproduction and Embryonic Development of the Orchid Dottyback (*Pseudochromis fridmani*)

3.3.2.1. Experimental Conditions and Broodstock Husbandry. This study was conducted at the Aquaculture Research Center (ARC) at The University of Maine campus in Orono, Maine. Three orchid dottyback (*Pseudochromis fridmani*) pairs were selected from a collection of spawning broodstock held by a commercial marine ornamental aquaculture company (Sea & Reef Aquaculture, LLC). Each pair had spawned regularly over the preceding year and had produced numerous viable embryos and larvae. Preceding the experiment, each pair was moved to an isolated 75L glass aquarium. The aquaria were bare, except for a few pieces of PVC pipe, which served as surrogate “dens” for the fish. The water temperature was maintained at $27 \pm 1^\circ\text{C}$, and salinity was kept at 31ppt. The photoperiod was 14h L: 10h D. Each tank was filtered by a submerged airlift sponge filter, which also served as the aquaria’s biofilters. Water quality parameters were checked daily and maintained at the following: pH, 8.1-8.3; NH_3 , 0.00-0.30mg/L; NO_2 ,

0.00-0.04mg/L. Approximately 25-30% of the tank water was replaced weekly using an artificial seawater mix (Forty Fathoms- Crystal Sea) combined with reverse osmosis filtered water. The new seawater was mixed and aerated for 24 hours prior to water exchanges. The fish were fed four times daily a complex “mixed” diet consisting of commercial aquarium diets, frozen mysid shrimp and fresh seafood. This feeding regime was identical to their prior regimen as commercial broodstock.

3.3.2.2. Copper Treatment. The pairs were held in their respective aquaria at 0.00mg/L Cu for 30 days prior to copper treatment (period 1). Copper (as copper sulfate) was administered, as a commercially available preparation (Red Sea “Paracure™”) to each aquarium at 0.10mg/L for a period of 21 days (period 2). Copper levels were tested twice daily on replicate (n=3) 10ml samples of aquarium water. Copper measurements were recorded by colorimetric analysis using a Lamotte “Smart Colorimeter™” utilizing the Diethyldithiocarbamate method (Lamotte #3646-SC). Copper levels were maintained at 0.10 ± 0.02 mg/L through twice daily doses of copper sulfate. Following the 21-day exposure period, each pair was held for an additional 30 days in copper-free water (period 3).

3.3.2.3. Sampling Procedures. Spawn quality information consisting of total number of eggs produced, percent normal embryos at 24h post-fertilization, percent normal embryos at 72h post-fertilization, percent hatch, and total number of larvae to survive 12h post-hatch was collected from each pair over the duration of the experiment. Three to four spawning cycles were recorded for each pair during each of the three treatment periods. Each pair was monitored daily for the production of eggs. Time of

spawning was recorded, and eggs were removed from the tank for initial analysis at approximately 24 hours post-fertilization.

The entire egg mass was removed from each aquarium and carefully blotted dry with lint-free paper. The egg mass was then transferred into a 25ml graduated cylinder containing a known volume of aquarium seawater. The amount of water displaced by the egg mass was recorded to the closest 0.10ml. Knowing the average volume per egg, this displacement method (Shirley, unpub. data) was used to calculate the approximate number of eggs produced per spawn. The egg mass was replaced after a small sub-sample of the egg mass (50-100 eggs) was removed at 24 and 72 hours post-fertilization and analyzed under a dissecting microscope.

The embryos were observed for any developmental abnormalities as compared to a “normal” developmental series (Fig. 3.2). The number of normal and abnormal embryos at each sampling session was recorded. On the evening of expected hatch (90 hours post-fertilization), the egg mass was removed from the aquarium to hatch in an 8 liter aquarium (Fig. 3.3). The egg mass was placed in a submerged 250ml Erlenmeyer flask and gently aerated with a rigid airline tube producing approximately 40 bubbles per minute. This motion facilitated hatch of the egg mass once the lights went out. At approximately 12 hours post-hatch, the surviving hatched larvae were euthanized with MS-222 (100mg/L) and counted.

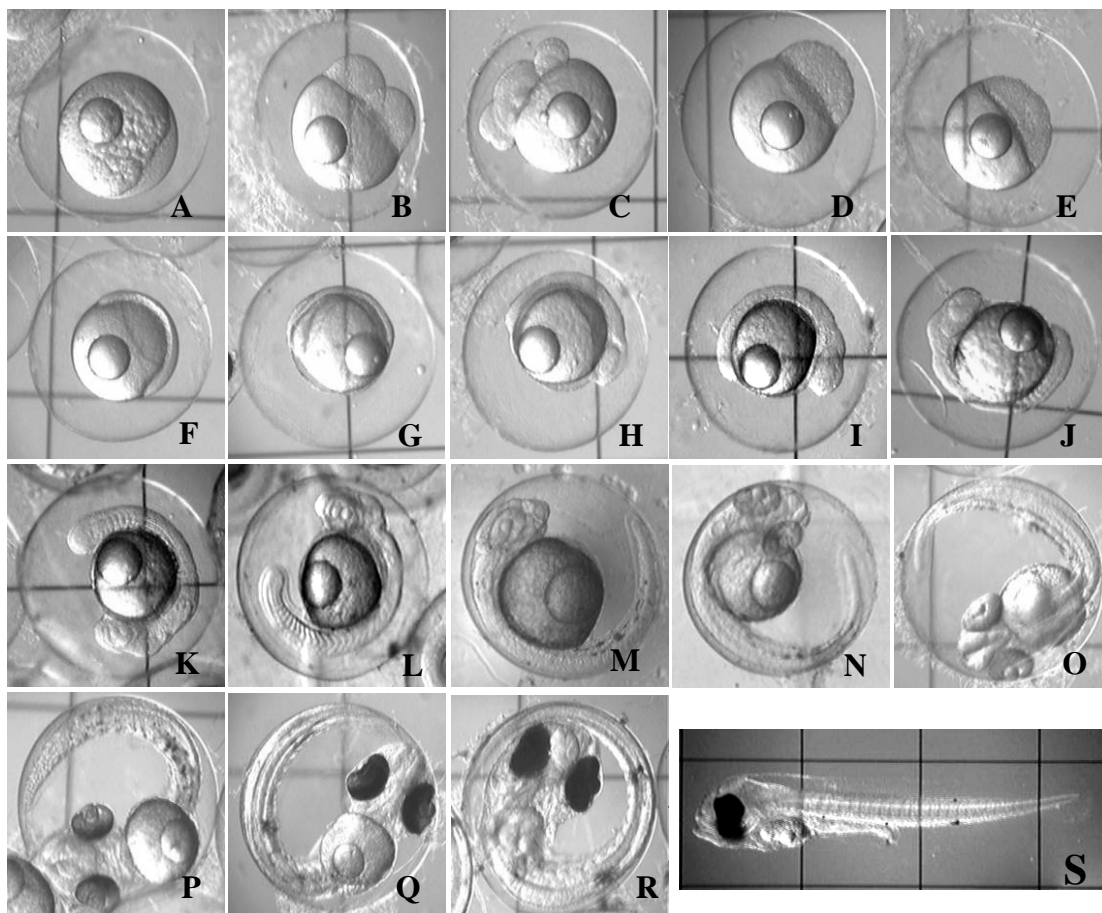


Figure 3.2. Photomicrograph series depicting the normal embryonic development of the orchid dottyback (*Pseudochromis fridmani*). Time = time post fertilization. A 20min; B 45min; C 90min; D 4h; E 8h; F 11h; G 14h; H 17h; I 19h; J 21h; K 24h; L 29h; M 34h; N 39h; O 45h; P 64h; Q 72h; R 91h; S 93h. Box grid on slide = 1mm.

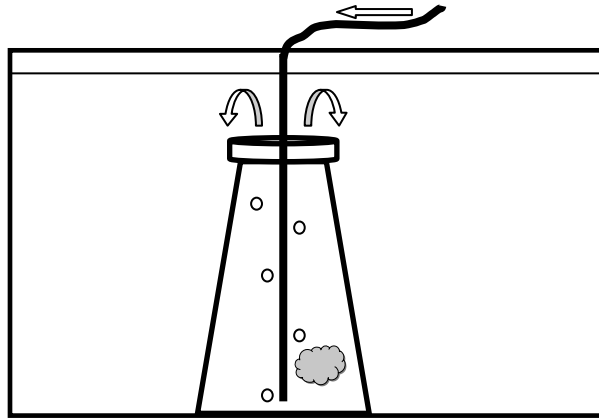


Figure 3.3. Schematic diagram of aquarium used to hatch dottyback eggs. Arrows indicate direction of air/water flow. The egg mass was gently aerated in a 250ml flask, submerged in an aquarium. Top of flask was open to allow hatched larvae to swim out.

3.3.3. Statistical Analysis

All data were analyzed using SYSTAT™ (ver 11.0). Data from Experiment 3 were analyzed using a one-way ANOVA with repeated measures. Normality of the data (Shapiro and Wilk, 1965) and homogeneity of the variance (Snedecor and Cochran, 1993) were tested to ensure assumptions for ANOVA were satisfied. Percent data were transformed (arcsine) before conducting analysis of variance. Tukey's HSD test (Snedecor and Cochran, 1993) was used to determine significant differences among the means ($p < 0.05$).

3.4. Results

3.4.1. Experiment 1

The daily mean concentration of copper measured for each treatment and the respective survival to 48 hours are presented in Table 3.1. Targeted copper concentrations

were rarely achieved and were difficult to maintain at the desired treatment levels for more than 8 hours. Generally, recorded copper levels were below the targeted values in the morning and above targeted values in the afternoon following re-dosing.

Additionally, fluctuating background levels of copper in our incoming seawater were recorded between 0.01-0.04mg/L, which further complicated proper dosing. Despite the fluctuations in copper levels, angelfish were acutely sensitive to copper at the higher doses. Flame angelfish in treatments that received the two highest levels of copper exhibited significant mortality within the first 12 hours of exposure.

Table 3.1. Mean concentration of copper exposure per treatment and percent survival of flame angelfish¹ following 48 hours of copper exposure during Experiment 1.

Treatment mg Cu/L	Mean Copper Concentration					Treatment Mean for Duration	STDEV	% Survival 48 hrs
	Day 1 AM	Day 1 PM	Day 2 AM	Day 2 PM	Day 3 AM			
0.00	0.01	0.04	0.00	0.04	0.00	0.02	0.02	100%
0.05	0.04	0.09	0.01	0.10	0.02	0.05	0.04	100%
0.10	0.04	0.14	0.06	0.15	0.04	0.08	0.06	80%
0.15	0.06	0.18	0.11	0.17	0.08	0.12	0.05	60%
0.20	0.13	0.22	0.12	0.22	0.05	0.15	0.07	60%
0.25	0.18	0.26	0.12	0.26	0.16	0.20	0.06	40%

¹ n=5 individuals for all copper exposed fish, n=3 individuals for control (0.00mg/L) treatment

The relationships between exposure duration and flame angelfish mortality at increasing copper concentration are illustrated in Figure 3.4. At 6 hours of exposure, the 0.25mg/L treatment had already experienced 40% mortality. At 12 hours of exposure, the 0.25mg/L treatment experienced a total of 60% mortality, whereas the 0.20mg/L and

0.15mg/L treatments experienced 20% mortality. After 24 hours, the 0.1, 0.15 and 0.20mg/L treatments experienced an additional 20% mortality. After 36 hours of exposure, the cumulative mortality had reached its maximum point and the values were constant until the conclusion of the experiment at 48 hours. All of the fish in the 0.00mg/L (n=3) and 0.05mg/L (n=5) treatments survived the trial. Results of a log-rank (Mantel-Cox) comparison of the survival curves revealed a significant difference ($p<0.05$) between the slopes of the 0.25mg/L and Control treatment curves. These data indicated that copper toxicity increased with increasing concentration and that mortality was strongly correlated with copper level. Figure 3.5 illustrates the relationship between the copper levels recorded and mortality in flame angelfish at the conclusion of this experiment.

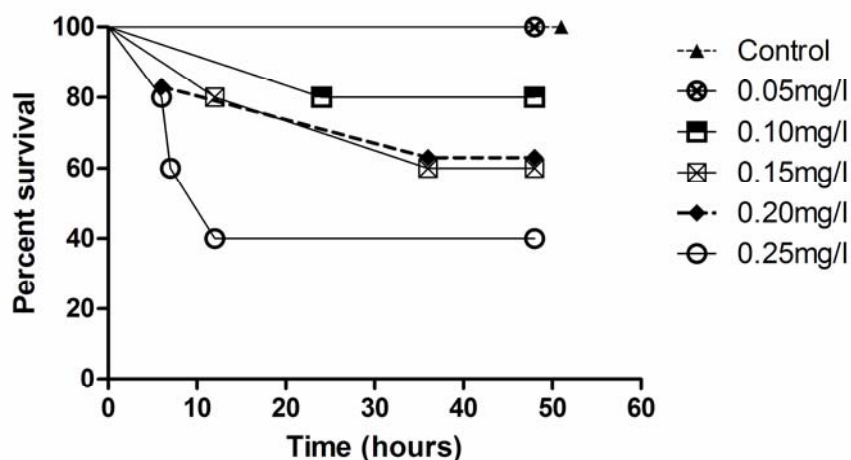


Figure 3.4. Survival of flame angelfish exposed to six levels of copper for 48 hours (Control n=3; all others n=5).

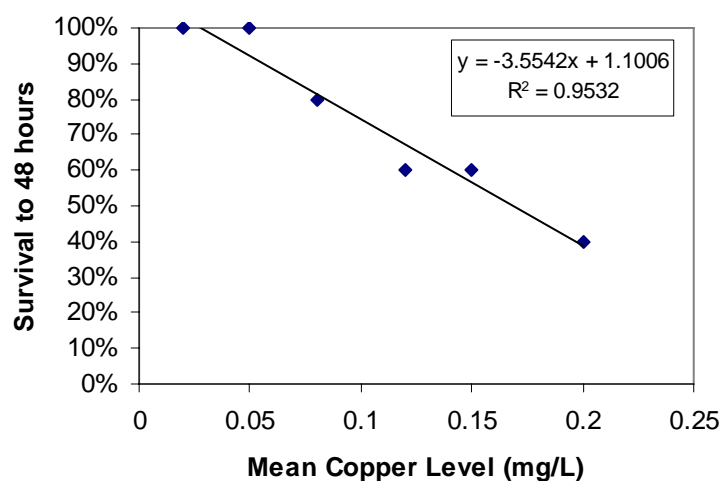


Figure 3.5. Relationship of flame angelfish survival to increased copper level after 48 hours of exposure. (0.00mg/L n=3; all others n=5)

3.4.2. Experiment 2

Copper levels were maintained at controlled levels in Experiment 2 and did not fluctuate significantly from the targeted treatment levels (Fig. 3.6). However, due to the use of the Dosatron™ on the flow-through seawater configuration, copper levels did not reach targeted concentrations until after 24 hours. We routinely measured values between 0.02-0.05mg/L of copper in the control treatment, indicating a background level of copper in our incoming source water. These background values were similar to what was observed in Experiment 1 (Table 3.1).

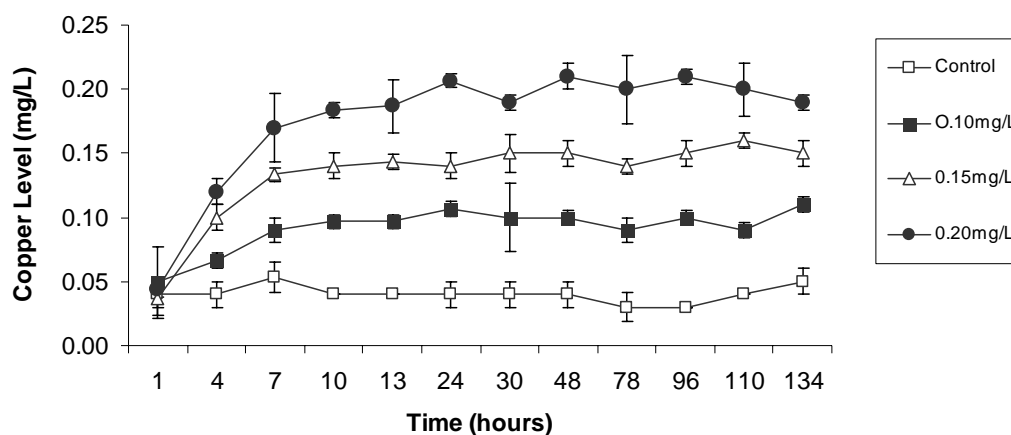


Figure 3.6. Recorded copper levels during trial Experiment 2. Copper levels were measured once each day on three replicate samples and values are reported as means \pm standard deviation.

The survival of flame angelfish exposed to the 4 levels of copper is shown in Figure 3.7. In contrast to what was observed in experiment 1, mortality was not observed until after 5 days of exposure at the highest level (0.20mg/L). After 144h of exposure (day 6), the 0.20mg/L treatment had experienced 25% mortality. No additional mortality was observed for the duration of the experiment. None of the other treatment groups experienced any mortality.

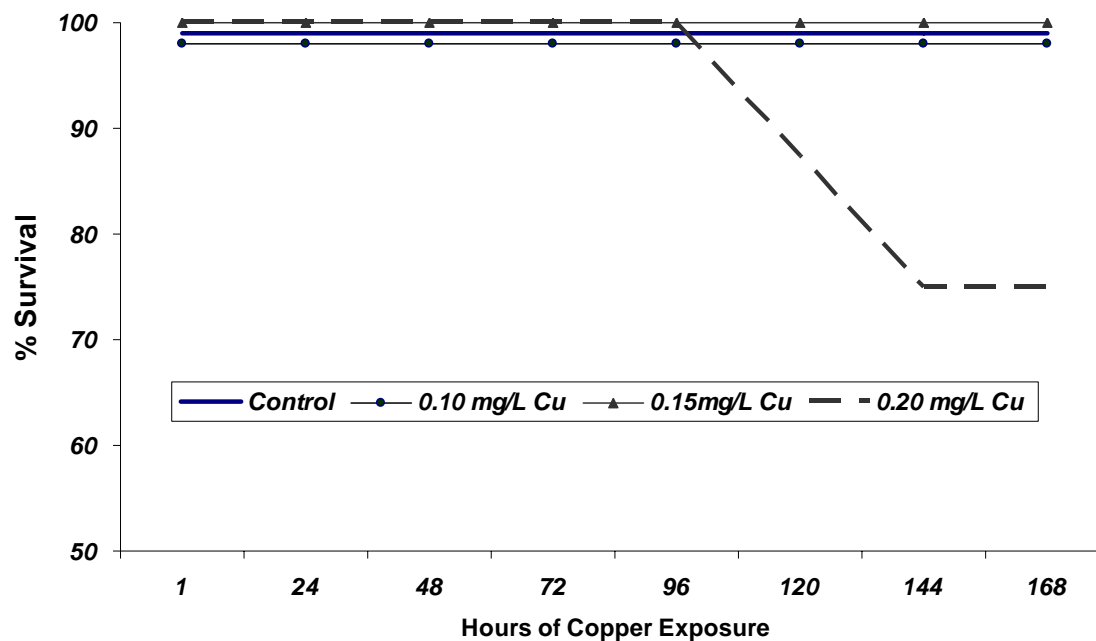


Figure 3.7. Survival of flame angelfish exposed to copper for 168 hours. Treatment levels were not reached until after 24 hours of dosing (n=8 fish per treatment).

3.4.3. Experiment 3

A summary of the data collected during experiment 3 is presented in Table 3.2. During the 30-day pretreatment period, each pair of dottybacks spawned between four and five times. The mean number of eggs produced per spawn (n=14) was 1927 ± 122 and the mean number of hatched larvae counted at 12 hours post hatch was 730 ± 135 . Normality of the developing embryos was 98% and 84% at 24h and 72h, respectively. Embryonic development closely followed the normal progression as depicted in Figure 3.2. The mean hatch rate was 46%. It is important to note that we believe the hatch rate was negatively affected by the artificial incubation of the egg mass, as typical hatch rates are normally near 100% when the egg mass was left under the male's care. Despite our

best efforts to replicate the motion and hatching environment of the egg mass, we typically could not achieve higher than 70% hatch rates.

Table 3.2. Summary of spawning data collected from orchid dottyback broodstock over the duration of Experiment 3.

	Period	# Spawns	Eggs/Spawn	Normal 24 hrs %	Normal 72 hrs %	Hatched Larvae	Hatch Rate %
Pair 1	Pre-Cu	4	2365 \pm 203	97.37 \pm 1	96.90 \pm 2	1107 \pm 46	54.07 \pm 6
Pair 2	Pre-Cu	5	2013 \pm 96	98.72 \pm 1	93.89 \pm 2	941 \pm 91	49.12 \pm 8
Pair 3	Pre-Cu	5	1491 \pm 122	99.13 \pm 1	60.97 \pm 23	376 \pm 193	33.59 \pm 19
Pair 1	During Cu	4	1305 \pm 66	56.73 \pm 9	0	0	0
Pair 2	During Cu	3	963 \pm 296	49.26 \pm 10	0	0	0
Pair 3	During Cu	2	746 \pm 186	37.78 \pm 7	0	0	0
Pair 1	Post-Cu	3	2641 \pm 55	95.30 \pm 2	91.58 \pm 3	1734 \pm 433	69.76 \pm 18
Pair 2	Post-Cu	4	1608 \pm 70	72.80 \pm 18	53.36 \pm 22	497 \pm 316	32.29 \pm 20
Pair 3	Post-Cu	0	N/A	N/A	N/A	N/A	N/A

Values are means \pm standard error.

During the 21-day copper treatment period (0.10mg/L Cu), the three pairs of dottybacks spawned 2, 3 and 4 times respectively. The mean number of eggs produced per spawn (n=9) was 1004 \pm 162, which was significantly fewer (p<0.05) than during both the pre-treatment and post treatment periods (Fig. 3.8A). Normality of the developing embryos (Fig. 3.8B) was also significantly lower at 24h (47%) and at 72h (0%) than during pre and post-treatment periods. The mean number of hatched larvae produced was 0, as all of the developing embryos had died by 72 hours post-fertilization.

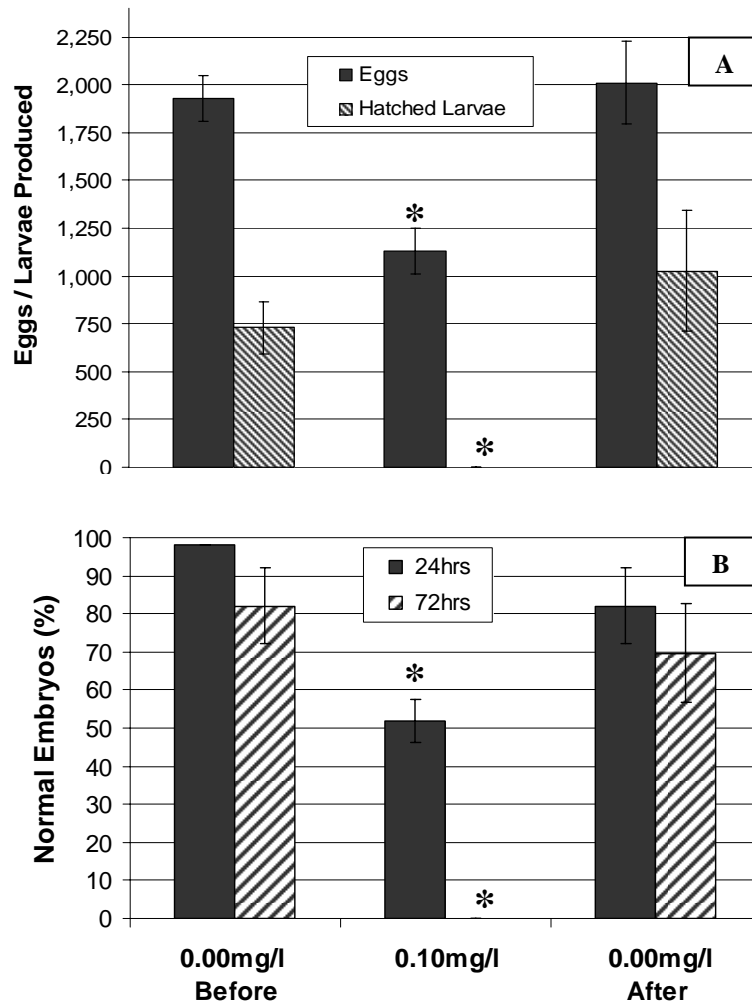


Figure 3.8. (A) Mean number of eggs and hatched larvae produced and (B) mean percent normal embryos at 24 and 72 hrs post fertilization, before, during and after copper treatment. Values are reported as means (n=3 pairs) \pm standard error. Asterisk indicates significant differences between means ($p < 0.05$).

Most of the abnormalities caused by the copper treatment were visible by 24h post-fertilization (Fig. 3.9). Exposure to copper at the levels tested caused many of the developing embryos to arrest shortly after fertilization. These arrested embryos would start to darken in color as they decomposed within the egg. Many of the eggs appeared less round, and in some cases were completely misshapen.

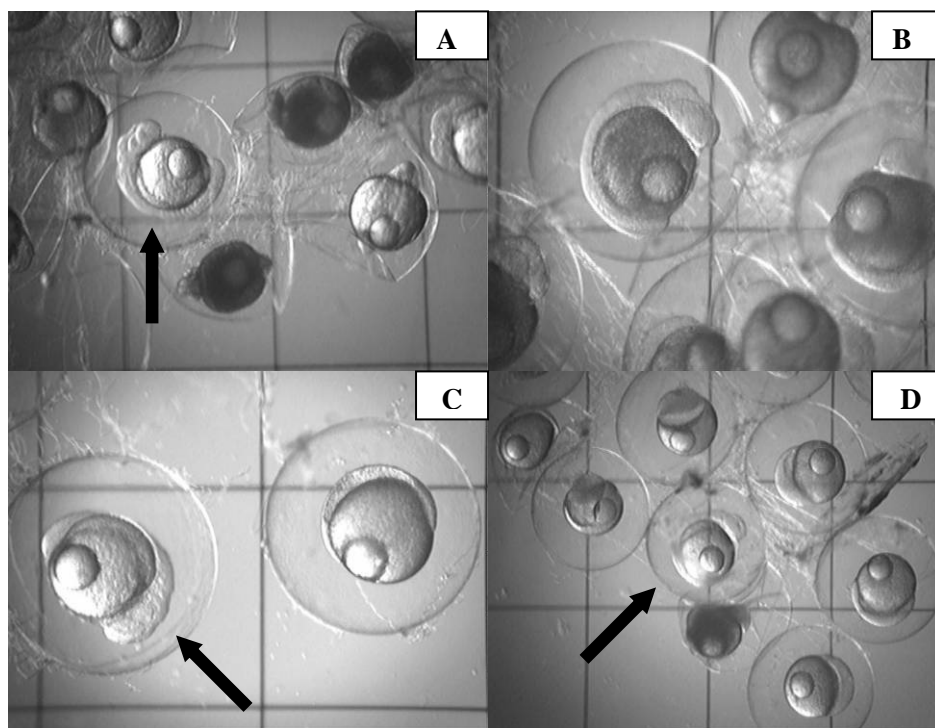


Figure 3.9. Photographs of abnormalities in early embryonic development of orchid dottyback eggs exposed to copper. Arrows indicate normal embryos. A) Normal 24hr post-fertilization embryo surrounded by dead (dark) and misshapen eggs. B) Close-up of arrested 24hr post-fertilization embryo, showing characteristic darkening of yolk area. C) Normal 24hr post fertilization embryo with embryo that arrested 10-12hrs post-fertilization. D) Normal 24hr post-fertilization embryo surrounded by embryos that arrested 4-8hrs post-fertilization.

Despite the large number of abnormalities observed at the 24 hours post-fertilization sampling period, many of the embryos continued to develop until about 30 to 40 hours post-fertilization. At the 72 hours post-fertilization sampling period it was clear that almost none (<1%) of the embryos exposed to copper sulfate had survived (Fig. 3.10).

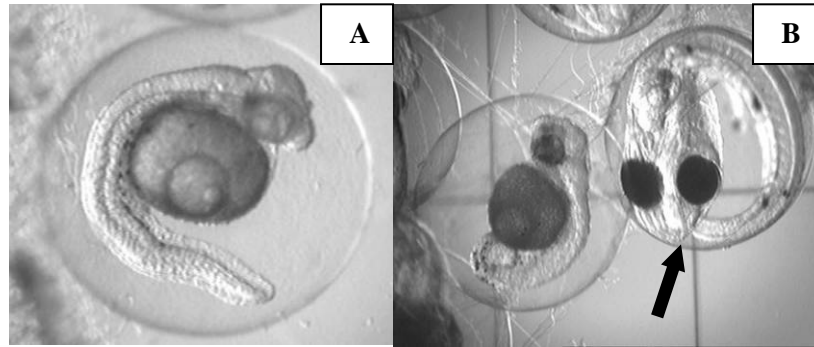


Figure 3.10. Photographs of abnormalities in late embryonic development of orchid dottyback eggs exposed to copper. Arrows indicate normal embryos. A) Close-up of arrested ~35hr post-fertilization embryo, showing darkening of yolk area and bent body axis. B) Normal 72hr post-fertilization embryo next to an embryo that arrested 30-40hrs post-fertilization.

Due to the fact that we allowed the male to care for the egg mass until just prior to hatch, very few eggs were available for sampling at the 72h sampling period during the copper treatment. It is common for the male to cull out dead or improperly developing eggs during the incubation period, thereby reducing the number of embryos we were able to evaluate. However, of the eggs remaining for sampling, we were still able to document abnormal development of embryos exposed to copper (Fig.3.10). It appeared that most development had ceased by 40 hours post-fertilization, despite normal development until that point. There were a very small (<1%) number of embryos that completed normal development to 72 hours post-fertilization. However, the male had consumed, or otherwise culled out, all remaining eggs from his den prior to the 90hr post-fertilization point when we would normally remove the egg mass for hatching. Therefore, there were no eggs available for hatching, resulting in zero hatched larvae during the copper treatment period.

During the 30-day recovery period (period 3), pair #1 and #2 spawned three and four times, respectively. The mean number of eggs produced per spawn ($n=7$) was 2050 ± 204 and the mean number of hatched larvae counted at 12 hours post-hatch was 1027 ± 318 . Normality of the developing embryos was 82% and 69% at 24h and 72h, respectively. Embryonic development closely followed the normal progression as depicted in Figure 3.2. The mean hatch rate was 48%. All spawning data collected in the post-treatment period were not significantly different from the pre-treatment period. However, pair 3 did not resume spawning during the 30-day post-treatment observation period. It is unknown why they did not resume spawning, as all other observed behaviors appeared normal.

3.5. Discussion

In the summer of 2004, acute mortality of flame angelfish broodstock and a gradual, but system-wide decline in reproductive performance (egg output and egg viability) across a range of other species (clownfish & dottybacks) was observed. After much investigation, it was determined that the only abnormal system parameter was an elevated copper level, caused by the introduction of copper wire into the sump of the recirculating holding system. The wire had slowly dissolved releasing copper into the water and eventually elevated the system level to 0.10-0.20mg/L. It was at least 6 weeks before the elevated copper level was discovered.

After much effort, the copper was removed from the system using a series of large water changes, removing all calcium carbonate substrate (which absorbed copper, acting as a copper sponge, subsequently re-releasing copper back into the water to equilibrium),

and by using a commercially available copper removing resin Cuprisorb™ (Seachem Laboratories, Inc.). Once the copper level was reduced to below 0.10 mg/L, most of the broodstock pairs began to spawn normally again.

The use of copper as a treatment for marine parasites requires constant exposure to levels between 0.15-0.25mg/L for a minimum of 21 days (Bassleer, 1996; Noga, 2000). Given the demonstrated sensitivity of angelfish to copper, copper treatment is counter indicated for this species. In strong contrast to the low-level treatments, fish in the highest-level treatments (Experiment 1) were immediately adversely affected by the addition of copper to the water. Within a few hours of exposure, many fish in the high-level treatments were exhibiting signs of stress (rapid respiration and erratic swimming behavior). The behaviors observed in these treatments were very similar to those observed during the acute mortality event at the ARC in Maine. It was clear that the addition of copper to the water at recommended therapeutic levels caused severe stress and injury to these fish.

Prior to the start of Experiment 1, mortality of two of the recently acquired fish was recorded. Those two fish had been observed showing signs of “stress” (rapid breathing and abnormal swimming behavior) upon arrival at OI. They were purposefully going to be assigned to the 0.00mg/L treatment group with the idea that any additional treatment “stress” might inadvertently cause mortality, confounding the effects of the copper treatment. However, mortality of these fish prior to the experiment caused the trial to commence with only 3 replicates in the control treatment.

This observed mortality could be attributed to the stress imposed on fish when they are recently imported. The only control treatment fish that were lost were the new

fish, acquired just prior to the experiment. Acclimation stress, and subsequent mortality, is unfortunately common in newly acquired fish. Mortality is particularly common with fish that are recently collected, as these undoubtedly were (coming directly from an importer). The remaining fish in the control treatment displayed no visible signs of stress and appeared completely normal for the duration of the trial. Also, the other newly acquired fish did not contribute disproportionately to the observed mortality in the other treatment groups. Furthermore, angelfish in the 0.05mg/L treatment did not experience any mortality, indicating that this species should not be negatively affected at the background levels of copper routinely measured in the incoming saltwater (derived from wells) at the Oceanic Institute.

In Experiment 2, the same acute onset of mortality following copper exposure was not recorded. However, flame angelfish at the highest treatment level did experience 25% mortality. It is possible that through the more gradual and constant addition of copper, these fish were able to somewhat acclimate to higher copper levels. More likely, as these fish are increasingly sensitive to copper at the higher treatment levels, the reduced fluctuation in copper dosing precluded exposure to these “upper maximums”. In Experiment 1, the targeted treatment levels were often exceeded in the afternoon, exposing the fish (even if only for an hour or two) to higher than desired levels. This exposure could likely have led to increased stress and reduced capacity for coping with the copper at lower levels and/or the ability to adjust to copper fluctuations. However, the former method of dosing copper (not using dosing equipment) is the most prevalent in the industry and is the method most readily utilized.

Prolonged exposure to copper, with reduced mortality, may be possible by utilizing a flow-through system with dosing equipment, such as the one described in Experiment 2. In this way, precise copper dosing at targeted therapeutic levels may be achieved. However, implementation of such systems is likely beyond the capacity of most hobbyists, importers or retail proprietors. Additionally, it is unknown whether prolonged exposure beyond the one-week end point of this trial would have lead to additional chronic mortality. It is also possible that other, less obvious, physiological injury could be caused by repeated or prolonged exposure to copper. Therefore, alternate treatment strategies should be investigated for the treatment of external parasites in this species of fish.

Results from Experiment 3 verify that the lowest level of copper observed during the period of accidental copper introduction (0.10mg/L) was high enough to impair the reproduction of marine ornamental fish broodstock. The effects of copper on reproduction in marine ornamental fish appear to be two-fold, negatively affecting both the adults and embryos. First, copper at 0.10mg/L affected the adult female orchid dottyback as evidenced by significantly reduced egg production. Second, the same copper level was toxic to the developing dottyback embryos, as none of the embryos survived past 48 hours of exposure. It is interesting to note that although the copper affected the adult females, the adult males did not seem to be affected to the same degree, as evidenced by unchanged fertilization rates of the eggs during copper exposure. Fertilization rates remained near 100% for the duration of this study and did not appear to be affected by copper levels in the water. It was observed that eggs produced in copper-treated water, if moved to copper-free water shortly after fertilization, would complete

normal embryonic development. This result is highly suggestive that the negative effects of copper on the developing embryo occurred after fertilization and were caused by exposure to copper in the water, rather than exposure inside the female during egg maturation.

Although copper exposure impaired the reproduction and embryonic development in this species, those effects were reversed shortly after returning the fish to copper-free water. Following the treatment, two of the three pairs immediately began producing eggs and larvae in similar numbers to pre-treatment levels. Although the third pair did not resume spawning during the 30-day, post-exposure evaluation period, that pair did begin spawning again shortly thereafter. These results indicate that moderate exposure to copper may not have negative long-lasting effects on reproduction in marine ornamental fish. However, caution must be utilized during therapeutic use of copper, as some species, such as flame angelfish, appear to be much more sensitive than others. From these results, it can be concluded that the recorded copper levels observed in the system at the ARC could have contributed to the flame angelfish mortality and prolonged reproductive decline in the other marine ornamental broodstock. Copper also proved very difficult to remove from recirculating systems and therefore should be avoided if other viable treatment alternatives are available.

3.6. Acknowledgments

Thank you to Søren Hansen, for assistance in identifying and correcting the copper contamination of our broodstock system. We wish to thank Dr. Michael Optiz for preparation of tissue samples and assistance in diagnosis of copper toxicity. Additional

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Chapter 4

DETERMINATION OF THE EFFECTS OF WATER SOURCE AND WATER TREATMENT ON FLAME ANGELFISH (*Centropyge loriculus*) REPRODUCTION

4.1. Abstract

This study was conducted at the Oceanic Institute (OI) to determine if water source and water treatment affected flame angelfish (*Centropyge loriculus*) reproduction. Flame angelfish broodstock pairs (n=5) were maintained and monitored for reproductive performance (fecundity, egg fertilization rates and egg viability) in either OI well-water (OIW), sterilized ocean water (OW) or OI well-water treated by recirculation (of biological filtration, protein skimming (foam fractionation), UV sterilization and mechanical filtration) (OI-RC). Results of this experiment indicated that although pairs maintained in OI well-water initially exhibited good spawning performance, fecundity, egg fertilization rates and egg viability declined after 25 weeks. Treatment of OI water by recirculation did not significantly improve flame angelfish reproductive performance, and fish in the OI-RC treatment exhibited only moderately improved fecundity compared to pairs in the OIW treatment. In contrast, pairs held in sterilized ocean water (OW) exhibited significantly greater fecundity, egg fertilization rates and egg viability from week 25 onward than pairs in either the OIW or OI-RC treatments. However, sterilization of ocean water by chlorine at levels > 25ppm (30 minutes) negatively affected flame angelfish egg fertilization rates indicating that alternative methods of water sterilization may be warranted.

4.2. Introduction

Research on the development of captive culture technologies for flame angelfish began at the Oceanic Institute (OI) in 1999 (Laidley, et al., 2004). During the first few years of activities, small numbers of fish were maintained and spawned utilizing water derived from OI saltwater wells (Laidley, et al., 2001; Laidley, 2003; Laidley et al., 2004). Early research quickly yielded promising results as angelfish were successfully spawned, and reared for the first time, utilizing the existing resources at OI (Laidley, et al., 2001). This success led to the initiation of some small-scale larviculture experiments over the course of the next few years (2000-2001). However, it quickly became apparent that the numbers of eggs generated by the few pairs of fish that OI was maintaining would not be sufficient to support research on a production scale. Therefore, a large-scale broodstock holding system was designed and built in 2002 to accommodate up to 18 pairs of flame angelfish (Fig. 4.1).



Figure 4.1. Photograph of a portion of the flame angelfish broodstock system at the Oceanic Institute. The system contained 19 identical, 1000L tanks that were configured into a recirculating system.

Construction of the system was completed in 2003 and was initially run as a flow-through system, as the previous angelfish tanks had in the past. However, it quickly became apparent, for some reason, that this configuration was not supporting the long-term health of the fish, as flame angelfish could not be successfully maintained in this system. Poor reproductive output and chronic mortalities of fish stocks severely impeded research efforts. It was thought that the observed negative effects on the fish could have been caused by the recorded relatively high total gas pressure, low pH and high CO₂ in OI well-water. To eliminate these undesirable water chemistry characteristics, the system

was converted to a recirculating system in late 2003. However, despite this modification, and the correction of the above water chemistry issues in the system, poor reproductive performance and chronic fish mortalities continued.

In February of 2005, after nearly a year of poor and inconsistent spawning performance by angelfish stocks in OI water on recirculation, researchers at OI switched water sources in the angelfish system from OI well-water to sterilized Pacific Ocean water, collected from the pier just across the street from OI. The results were immediate and dramatic. Flame angelfish egg fertilization rates went from nearly zero to over 60% (Fig. 4.2). Additionally, total egg production, as well as overall egg quality, increased steadily subsequent to this transition (Fig. 4.3).

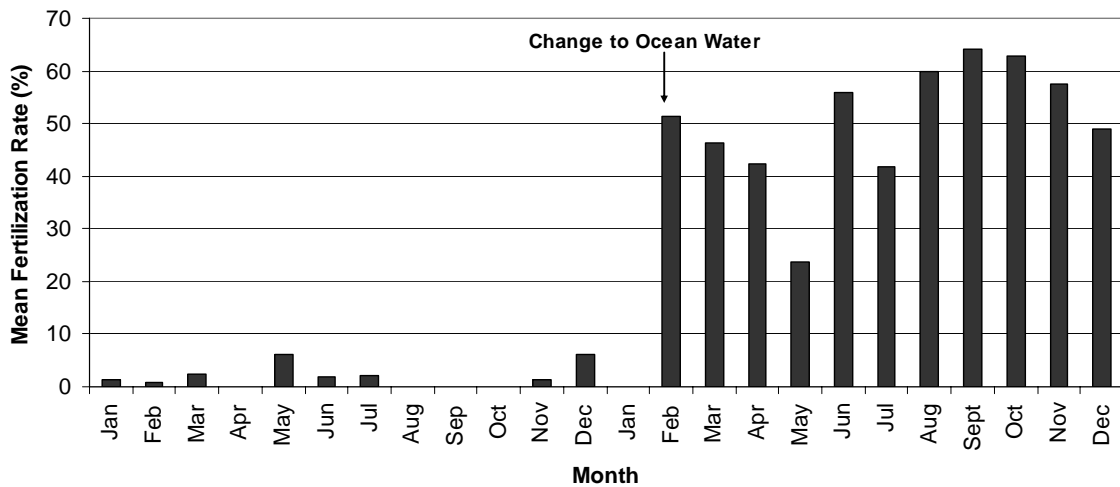


Figure 4.2. Mean fertilization rates of flame angelfish eggs from January 2004 through December 2005 (n=12 pairs). Arrow indicates change from OI well water to sterilized ocean water from Makai Pier in Feb. 2005.

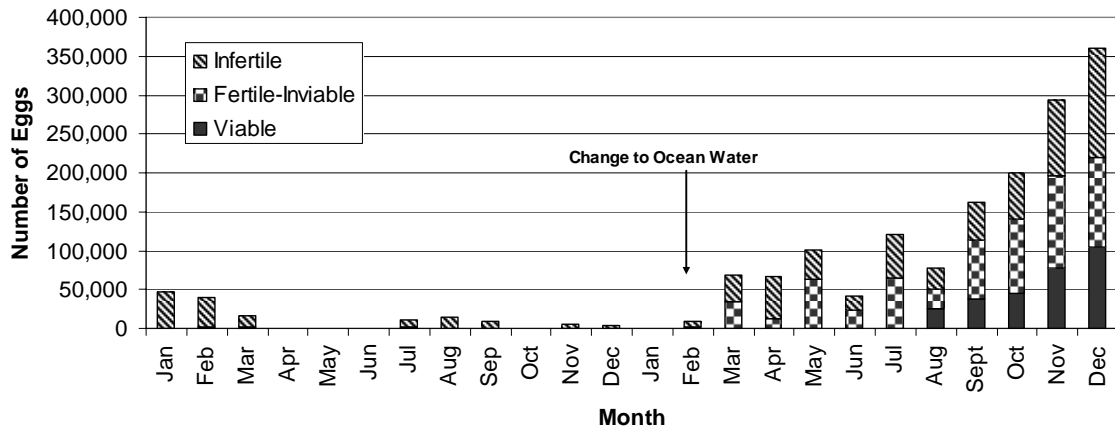


Figure 4.3. Flame angelfish total monthly egg production for 2004 and 2005 (n=12 pairs). Eggs were characterized as infertile, fertile – inviable, or viable. Arrow indicates change from OI well-water to sterilized ocean water from Makai Pier in Feb. 2005.

These results suggested that water chemistry may be fundamentally important in flame angelfish egg production and could potentially mask any future treatment effects we planned to investigate. However, the preceding results were potentially confounded due to the fact that some new pairs were brought in at the time of the water transition, and therefore resulting effects were being observed on a mixture of both new and old pairs. In order to determine if there was a significant effect of water source on egg quality, a replicated trial was planned to test OI well-water vs. sterilized ocean water. Additionally, OI well-water treated by recirculation would be tested to determine if further treatment of OI well-water could support angelfish in reproductive condition. The objectives of this trial were to test the effects of water source, as well as water filtration, on flame angelfish egg production and egg quality in order to optimize egg production from OI flame angelfish broodstock populations.

4.3. Methods

4.3.1. System Configuration

This experiment was conducted in the indoor marine ornamental system (IMO) at the Oceanic Institute. The system was originally configured as a recirculating system, comprised of fifteen 750L blue, polyethylene tanks. The system incorporated a fluidized-bed sand biological filter, protein skimmer, ultraviolet sterilizer and heat pump into a full, plug-flow design. This ensured that all of the water that left the tanks passed through all of the filtration components, respectively, prior to its return to the tanks. A photograph of the tanks within the system can be seen in Figure 4.4. The filtration components can be seen in Figure 4.5.



Figure 4.4. Photograph of a portion of the Indoor Marine Ornamental (IMO) system at the Oceanic Institute. The system contained 15 (750L) tanks, which were connected to a recirculating filtration system. Ten of the tanks were modified, by the addition of a bio-filter and pump, to run as individual systems.

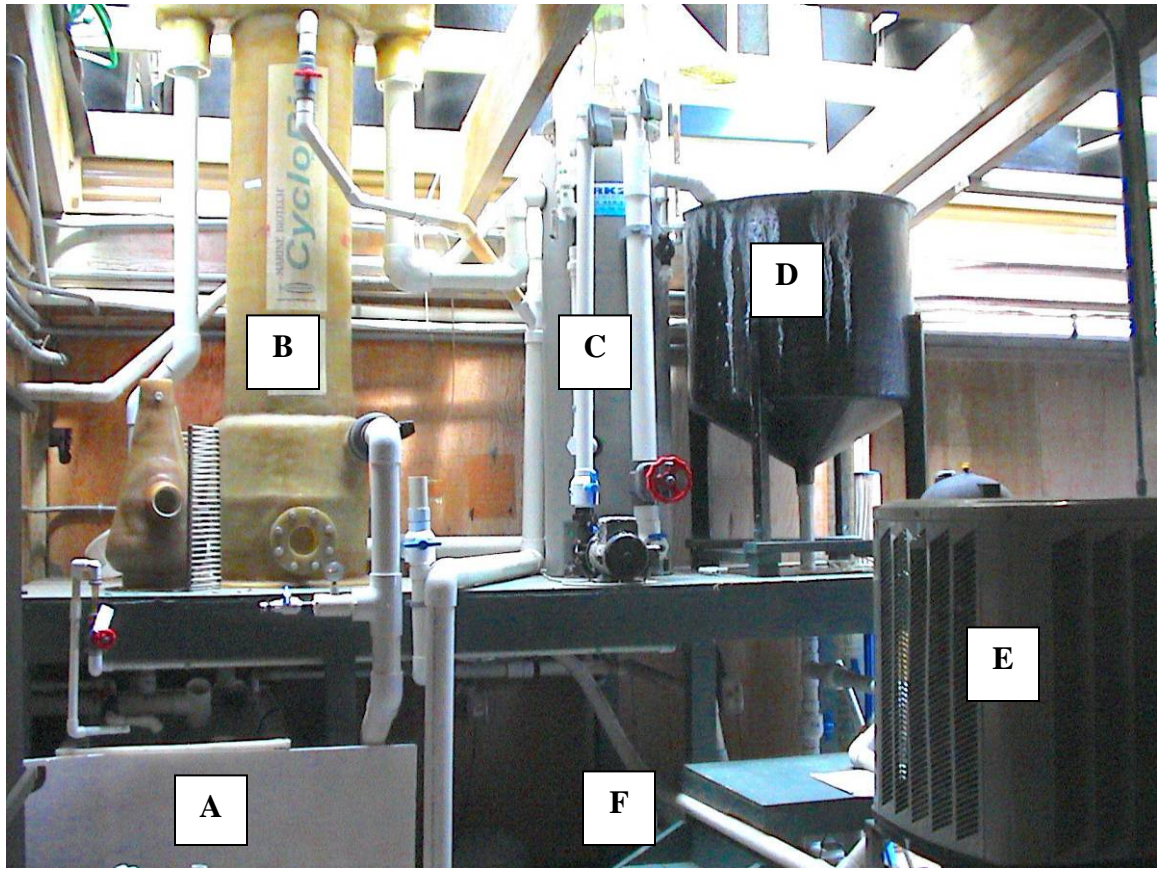


Figure 4.5. Photograph of the Indoor Marine Ornamental (IMO) filtration system at the Oceanic Institute. Water leaving the tanks in Figure 4.4 terminated into the main sump (A). From there, water was pumped to the biological filter (B). Gravity flow forced the water from there to the protein skimmer (C), which emptied into a head tank (D). Gravity flow then directed the water to a heat-pump (E) and then through the UV sterilizer (F). From there, the water was directed back to the tanks. This design allowed for all the water to be treated by all the filtration components without any by-pass or side-loops.

The system was temporarily modified to allow for ten individually replicated 750L tanks to be compared to five 750L tanks on the main recirculation system. Three treatments were randomly assigned to 15 tanks ($n=5$) as follows. Five of the individual tanks were supplied with sterilized ocean water (OW) and each was maintained with an individual canister biological filter. Five tanks were supplied with OI well-water (OIW) and each was maintained with an individual canister biological filter. The remaining five

tanks were left as originally configured on the recirculation system and were filled with OI well-water (OI-RC). A diagram depicting the configuration of the modified tanks and egg collection is presented in Figure 4.6.

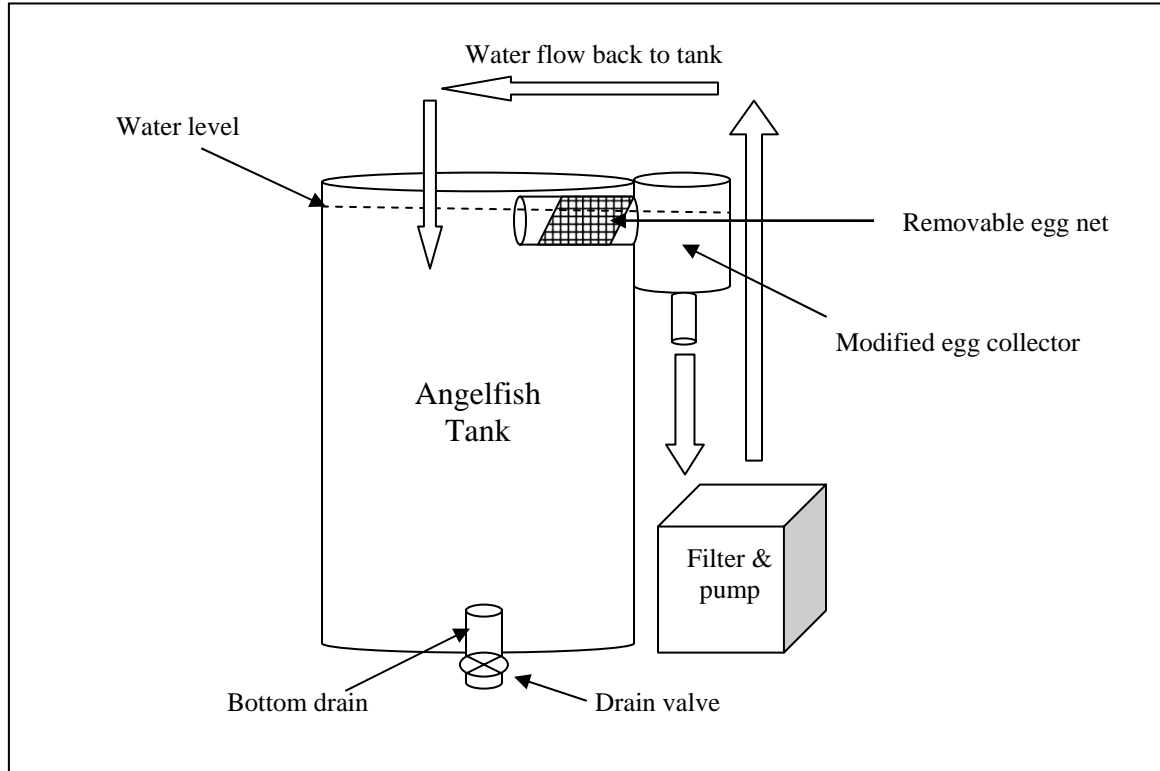


Figure 4.6 Schematic diagram of modified Indoor Marine Ornamental (IMO) system tanks at the Oceanic Institute. Ten of the tanks were modified to run as individual systems. Each tank was fitted with an individual bio-filter/pump combination, which was supplied by water leaving the egg collector. Water was then filtered and pumped back to the tank through a submerged spray-bar. Eggs were collected in a removable, submerged net that was placed in front of the modified egg collector.

4.3.2. Fish Acquisition, Quarantine and Conditioning

Angelfish were obtained from a commercial supplier (Hawaiian Sealife Inc., Honolulu, HI.) as individuals and paired according to external characteristics (see Chapter 2). Upon arrival at OI, fish were placed into 20L buckets and acclimated to the

water conditions on site. After acclimation, fish were treated with hydrogen peroxide at 100ppm for 45 minutes, followed by a 2-minute freshwater dip to exclude any potential external parasites. Fish were then quarantined in isolated, bare, 75L glass aquaria for a period of 4 weeks. Aquaria were supplied with approximately 400ml/minute of OI well-water (32ppt, 26.5°C), which provided for 7-8 tank exchanges per day. Additionally, tanks were equipped with external filters to provide additional circulation and water filtration. Lighting was provided by fluorescent bulbs for 12 hours daily, in addition to the indirect sunlight received. PVC pipes and fittings were provided as hiding places for the fish and each tank of fish was fed twice daily with high-quality commercial aquarium diets.

During the quarantine period, angelfish were treated every three days for the first 2 weeks with the same treatment protocol as described above (100ppm hydrogen peroxide/freshwater dip) to prevent or reduce the occurrence of external parasite infections. Following treatment, fish were moved to clean aquaria, which helped to break the life cycle of any potential parasites. This treatment protocol worked very well, as we rarely observed any parasite outbreaks, and the procedures were well tolerated by the fish. After the initial two-week quarantine period, fish were observed without subsequent treatment for an additional two weeks. If no further treatments were required (no signs of bacterial or parasite infections) after the entire 4-week period had lapsed, fish were considered ready for stocking into our experimental system.

4.3.3. Ocean Water Sterilization Protocol

Ocean water (35ppt) was pumped from approximately 100ft offshore (20ft deep) through a 5-micron filter into a 1,000L tank and chlorinated at 60ppm (sodium hypochlorite) prior to being brought onto OI campus. The water was chlorinated for 24 hours prior to being neutralized with sodium thiosulfate. This water was held in an aerated 10,000L tank that was continuously pumped through a 10-micron cartridge filter and 120 watt UV sterilizer. This water was then pumped to the tanks of the OW treatment group. The chlorination protocol was later modified several times in attempts to overcome deteriorating coastal water quality (due to heavy rains) and to satisfy increasing bio-security concerns that warranted higher chlorination levels for longer durations. Eventually, a protocol was set to maintain a chlorination level of 25ppm for 30 minutes after determining that this level would satisfy OI bio-security protocols and meet our sterilization requirements.

4.3.4. Experiment Stocking and Environmental Conditions

Following the quarantine period, flame angelfish pairs were stocked (1 pair per tank) into their respective treatments (ocean water = OW, OI well-water = OIW, or OI well-water on a recirculating system= OI-RC). Additionally, 2 new pairs replaced existing pairs in each treatment group following the final change in chlorination protocol to ensure equal representation of “old” and “new” pairs in each group. All of the tanks were kept bare except for an artificial “reef” made out of PVC scaffolding, which served as hiding places for the fish. Lighting was provided by overhead fluorescent lights, which were controlled by a dimmer to simulate dawn and dusk periods. Day length was fixed at

14hL:10hD. Temperature of the individual tanks was controlled by a room air conditioner, which maintained the water temperature at 26-27°C. Each week, approximately 25% of the tank volume was replaced with new saltwater (from the appropriate source) in the OW and OIW treatments. The OI-RC treatment (main recirculating system) received approximately 10% turnover daily through the continuous, slow addition of new OI well-water into the system's sump. In all treatments, water quality parameters (temperature, salinity, DO, pH, NH₃, NO₂, and NO₃) were monitored weekly and additional water changes were performed if water quality deteriorated.

4.3.5. Broodstock Husbandry

Broodstock pairs were fed four times daily. Their feeding regime consisted of two daily feedings of a “raw” mixed diet and two daily feedings of various commercial aquarium diets. The “raw” diet (Table 4.1) was comprised of squid, shrimp, fish eggs, spinach, peas, and dried algae (Nori). Ingredients were prepared in a blender and the mixture was kept frozen until fed. The tanks were routinely siphoned to remove any excess feed or debris and the tank walls and bottoms were scrubbed with a soft pad on a long pole once weekly. Additionally, the PVC scaffolding, which served as an artificial “reef” for the fish, was removed once per week and cleaned thoroughly. Fish were not removed from the tanks during cleaning.

Table 4.1. Composition of “Raw” control diet used for marine ornamentals at the Oceanic Institute¹.

Ingredient	% of Diet
Squid	27.71
Shrimp	27.71
Fish Eggs	18.48
Spinach	11.55
Peas	11.55
Nori	3.00

¹All ingredients were stored frozen. The squid, peas, spinach and nori were purchased from local supermarkets. Shrimp were provided by the OI shrimp department and the fish eggs were obtained from OI broodstock Pacific threadfin (*Polydactylus sexfilis*) or bluefin trevally (*Caranx melampygus*) surplus spawns.

4.3.6. Egg Collection and Sampling

Each morning, all of the nets that were on the surface drains from the tanks were removed and rinsed into individual, clean 1L beakers (containing approximately 800ml of clean seawater). If eggs were present, the beaker was placed under moderate aeration for a few minutes to homogeneously mix the eggs. While under aeration, five separate 10ml samples were taken and combined. Once all the samples were collected, they were poured into wells of a 12-well counting tray. The eggs were then counted under a dissecting scope and characterized as, 1) infertile (no clear cell division or other evidence of fertilization), 2) fertile-inviable (clear cell division but abnormally developed or development had arrested), or 3) viable (containing a fully developed embryo, ready to hatch). This procedure was repeated for each sample taken. Images of representative eggs in each of the above categories are presented in Figure 4.7.



Figure 4.7. Photographs of representative egg quality categories for flame angelfish eggs. (A) Unfertilized egg (B) Fertile-inviable egg (arrested early) (C) Fertile-inviable egg (arrested late) (D) Viable egg

4.3.7. Statistical Analysis

All data were analyzed using SYSTAT™ (ver 11.0). Weekly means were compared between treatments using two sample *t*- tests. Differences among the means were considered significant if $p < 0.05$.

4.4. Results

4.4.1. Effects of Ocean Water Chlorination Protocol on Flame Angelfish Egg Fertilization

Figure 4.8 illustrates the weekly mean fertilization rates ($n=5$ pairs) of flame angelfish following transitions between ocean water chlorination protocols. When OI originally started using ocean water for the flame angelfish systems (Feb. 2005), the water was chlorinated to 25ppm for 30 minutes. Near the start of the current trial, (March, 2006) Hawaii experienced record, nearly continuous rainfall over 40 days, which dramatically affected the near-shore water quality. Increased nutrients from freshwater run-off caused bacteria levels in the coastal waters to surge. Therefore, when the experiment first began, (week 1-6) the ocean water chlorination protocol was modified to

provide sufficient sterilization for this higher bacteria level. The chlorination protocol at the start of this trial required the water to be treated at 60ppm for 24 hours.

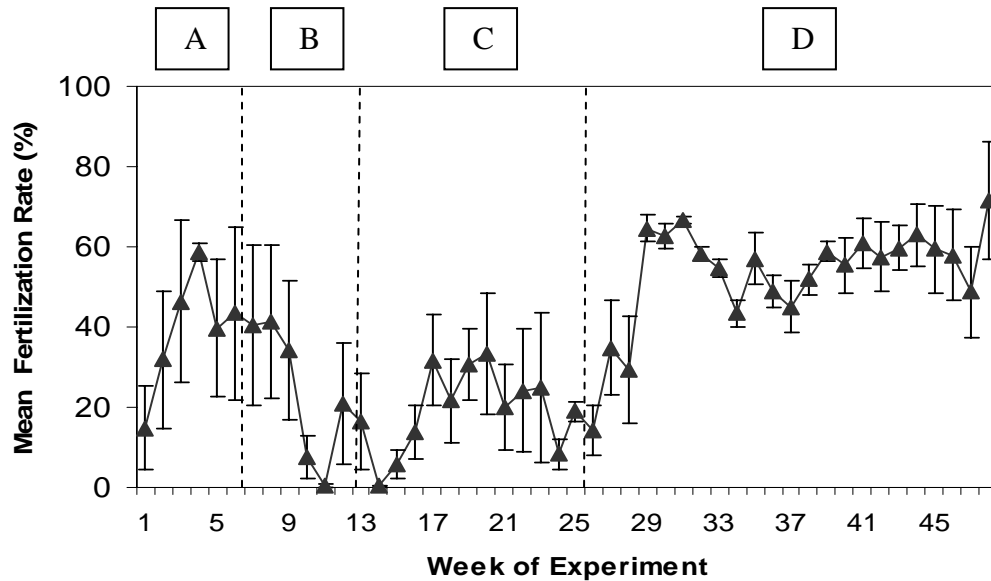


Figure 4.8. Flame angelfish weekly mean fertilization rates while held in ocean water treated with different levels of chlorine. Data are presented as weekly means (n=5 pairs) \pm standard error. Dotted lines indicate change in chlorination protocol. Water was treated with chlorine to 60ppm for 24h (Period A), 200ppm for 24h (Period B) and 25ppm for 24h (Period C) and 25ppm for 30 minutes (Period D).

Initially, fertilization rates among OW treatment pairs were highly variable and uncharacteristically low at approximately 40%. Increased bio-security concerns at OI brought about by the large quantities of raw ocean water being brought onto campus resulted in another modification of the ocean water chlorination procedure. At week 6, the chlorination protocol was changed to 200ppm for 24 hours. Following this change, we observed a dramatic decline in fertilization rate, as mean weekly fertilization rates approached zero. During week 12, the chlorination level was reduced to allow for 25ppm chlorine for at least 24 hours. Following this change, we observed a rapid, but temporary, increase in egg fertilization rate from weeks 15-21, followed by a decrease from week 21

to 23. After realizing only moderate gains in egg fertilization rates, the chlorination level was further reduced to the original protocol of 25ppm for 30 minutes (week 25). Following this change in protocol, and a complete (100%) water exchange in the tanks, we immediately observed a rapid increase in fertilization rate (week 25-29). Following this increase in fertilization rate, weekly mean fertilization rates remained above 40% and leveled off near previously recorded (Feb. 2005- Dec. 2005) values of approximately 60%.

4.4.2. Effects of Water Source on Fecundity

All pairs began spawning shortly after the experiment commenced. Furthermore, since we identified potentially confounding effects of chlorination protocol on egg fertilization rate, the experiment was restarted from the time the chlorination protocol was switched to 25ppm for 30 minutes and fish began demonstrating expected fertilization rates (week 27). However, only four of the five pairs in the OW and OIW treatments spawned consistently following the re-start of the trial. Therefore, only data from pairs that continued to spawn throughout the entire trial period were included in the following results.

Results from the first half of the experimental period (weeks 1-24) indicated that egg output was initially greater in the OIW treatment than in the OW treatment (Fig. 4.9). However, from week 25 onward, fecundity among pairs in the OIW treatment dropped dramatically. This decline continued to week 42, when all pairs in this treatment ceased spawning regularly. In contrast to the OIW treatment, fecundity among pairs in the OW treatment increased over the duration of the study. From week 27 onward, fecundity

among OW pairs significantly increased, while fecundity among OIW pairs significantly decreased. From week 39 onward, fecundity was significantly greater ($p<0.05$) among OW pairs than OIW pairs.

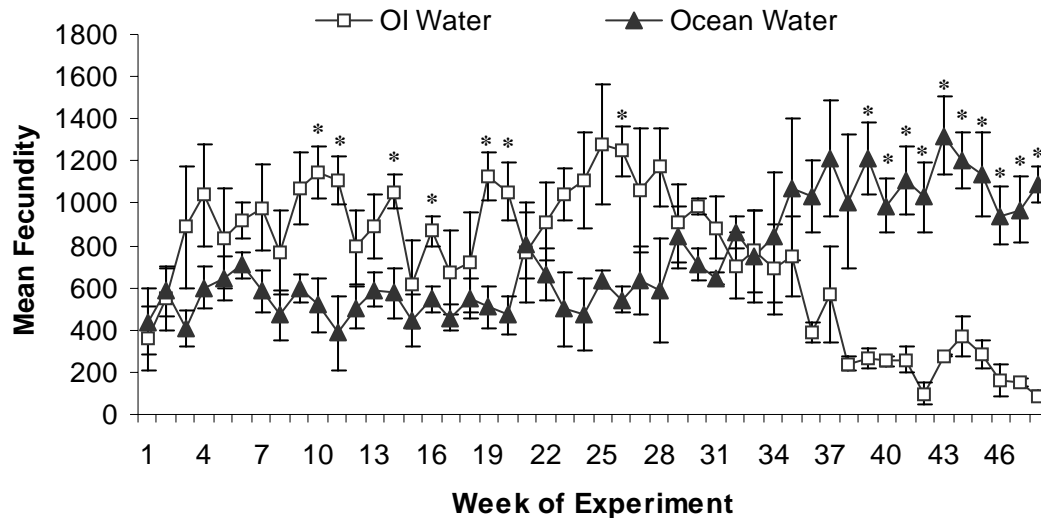


Figure 4.9. Flame angelfish mean fecundity (number of eggs per spawn) while held in OI water vs. sterilized ocean water. Data are presented as weekly means ($n=4$ pairs) \pm standard error. Asterisk indicates significant difference ($p<0.05$).

4.4.3. Effects of Water Source on Egg Fertilization

For the first 29 weeks of the trial, mean fertilization rates were not significantly different between the OW and OIW treatments, though pairs in OIW treatment appeared to have slightly higher fertilization rates from week 14 to 28 (Fig. 4.10). As with fecundity, mean fertilization rates dropped quickly in pairs held in OI water after the first 25 weeks of the trial. In contrast, pairs in ocean water exhibited a sharp increase in mean fertilization rate beginning at week 25 following the transition back to the original chlorination protocol. From week 25 onward, OW treatment pairs exhibited fairly

consistent weekly mean fertilization rates of nearly 60%. Mean fertilization rates from pairs in the OW treatment were significantly greater than pairs in the OIW treatment from week 30 onward.

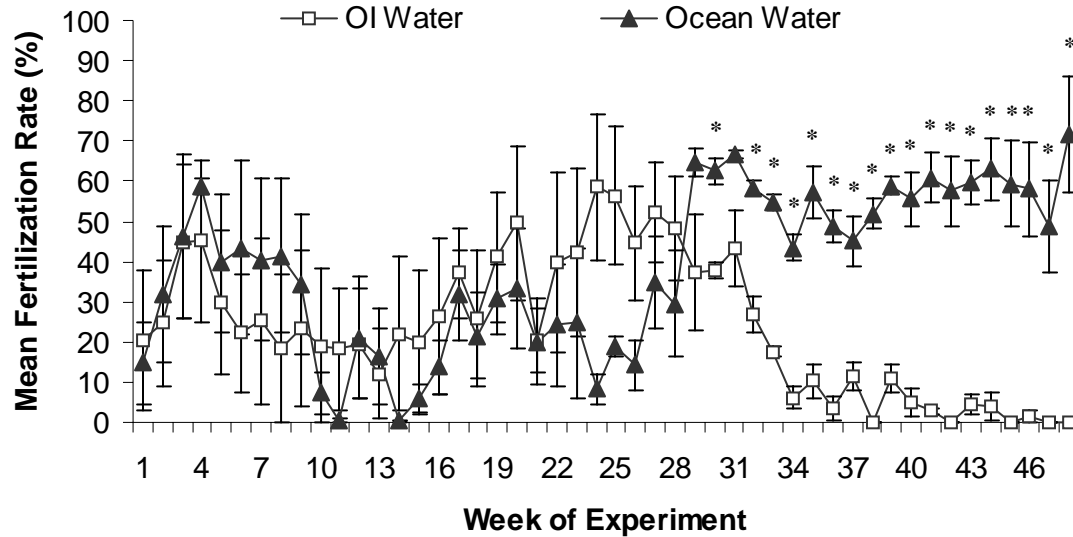


Figure 4.10. Flame angelfish mean fertilization rates while held in OI water vs. sterilized ocean water. Data are presented as weekly means ($n=4$ pairs) \pm standard error. Asterisk indicates significant difference ($p<0.05$).

4.4.4. Effects of Water Source on Egg Viability

The effects of water source on weekly mean egg viability rates are presented in Figure 4.11. For the first 33 weeks, mean egg viability rates were not significantly different between the OW and OIW treatments. However, OW treatment pairs appeared to have slightly higher viability rates from week 3 to 9, while OIW treatment pairs demonstrated slightly higher viability rates from week 19 to 28. However, that trend did not persist, as egg viability among OW pairs rapidly increased from week 27 onward, while egg viability in pairs in OI water rapidly decreased during the same period.

As observed with fertilization rates, mean egg viability rates from pairs in the OW treatment were significantly greater than pairs in the OIW treatment from week 34 onward.

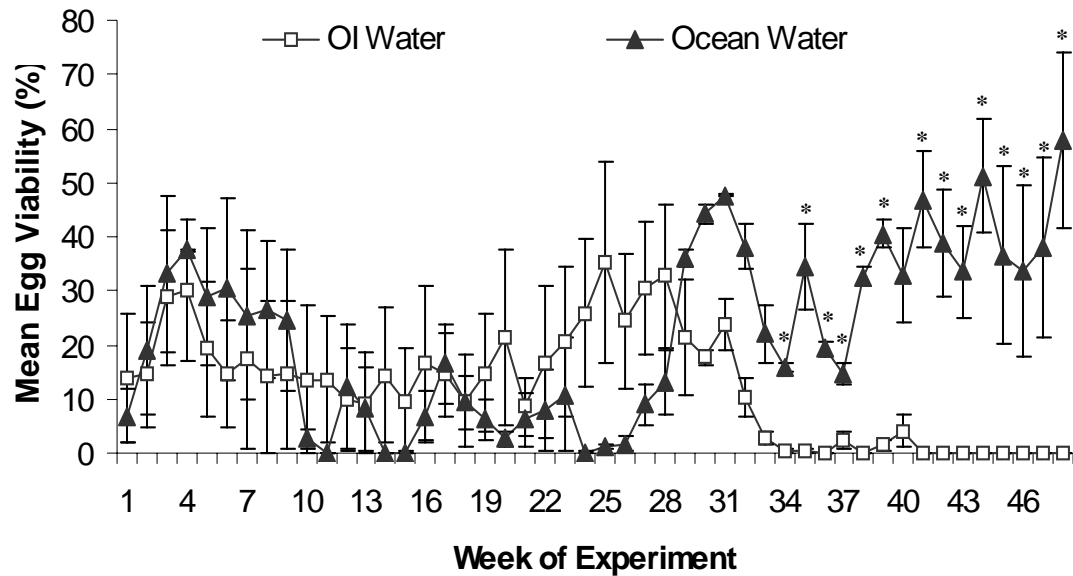


Figure 4.11. Flame angelfish mean egg viability while held in OI water vs. sterilized ocean water. Data are presented as weekly means ($n=4$ pairs) \pm standard error. Asterisk indicates significant difference ($p<0.05$).

From week 31 to week 37, viability of eggs declined among OW treatment pairs. Weekly mean egg viability rates dropped from nearly 50% to 15% despite relatively consistent fertility rates over the same period. However, despite this decline, egg viability rates continued to be significantly greater in pairs from the OW treatment than pairs in the OIW treatment from week 37 onward. Egg viability in pairs held in ocean water rapidly returned to more “normal” levels from week 38 onward, indicating that whatever caused the decline was a temporary effect.

4.4.5. Effects of Treatment of OI Water on Flame Angelfish Reproduction

4.4.5.1. Effects of Water Treatment on Fecundity. Weekly mean egg

production from five pairs held in OI water, treated by use in a recirculation system (OI-RC) compared to the pairs held in untreated OI well-water (OIW) is presented in Figure 4.12. In contrast to what was observed in the OIW treatment, where fecundity increased for the first 25 weeks followed by a significant decline, mean weekly fecundity among OI-RC pairs remained relatively constant throughout the duration of the experimental period. Pairs in the OI-RC treatment group produced approximately 700 eggs per spawn daily over the duration of the experiment and, during weeks 42 and 45, produced significantly greater numbers of eggs per spawn than pairs in the OIW treatment.

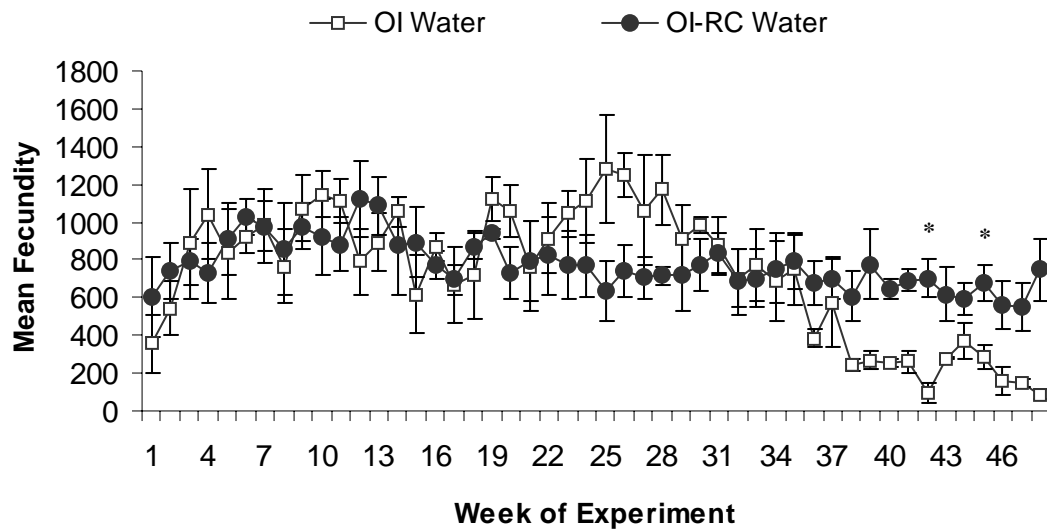


Figure 4.12. Flame angelfish mean egg production in OI water treated by water recirculation (OI-RC) vs. untreated OI water (OIW). OI-RC water was treated via continual passes through a biological filter, foam fractionator and ultraviolet sterilizer. Data are presented as weekly means ($n=5$) \pm standard error. Asterisk indicates significant difference ($p<0.05$).

4.4.5.2. Effects of Water Treatment on Fertilization Rate. Weekly mean egg fertilization rates from five angelfish pairs held in OI water treated by use in a recirculation system (OI-RC) compared to the pairs held in untreated OI water (OIW) are presented in Figure 4.13. Mean weekly fertilization rates slowly declined among OI-RC pairs over the course of the study from 40% to approximately 20%, whereas OIW pairs exhibited a brief period of increased fertilization rates (approaching 60%) followed by a dramatic decline to nearly zero. From week 38 onward, OI-RC pairs exhibited higher mean fertilization rates (~15-20%) than pairs from the OIW treatment (~0-2%). However, mean egg fertilization rates were not significantly different from OIW for the duration the experimental period.

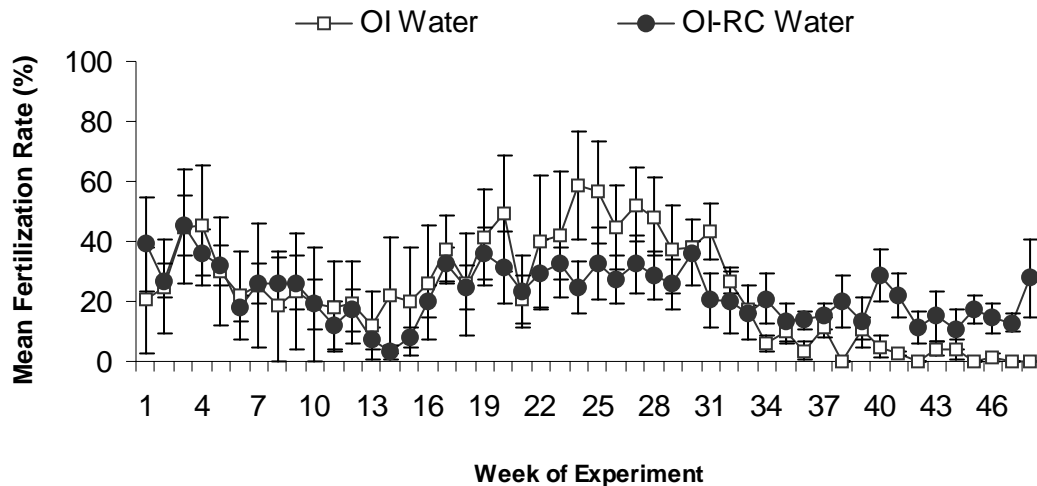


Figure 4.13. Flame angelfish weekly mean egg fertilization rates in OI water treated by water recirculation (OI-RC) vs. untreated OI water (OIW). OI-RC water was treated via continual passes through a biological filter, foam fractionator and ultraviolet sterilizer. Data are presented as weekly means ($n=5$) \pm standard error.

4.4.5.3. Effects of Water Treatment on Egg Viability. Mean egg viability rates of the five angelfish pairs from the OI-RC treatment compared to the OIW treatment pairs are presented in Figure 4.14. Weekly mean egg viability was not significantly different between pairs from the OI-RC and OIW treatments and declined over the duration of the study from 30% to approximately 5%. OIW treatment pairs exhibited a brief period (5 weeks) of relatively high (30%) mean egg viability from week 23 to 28. However, mean egg viability rates among the OIW treatment rapidly dropped to zero from week 28 to 33 and remained at zero for the remainder of the experiment.

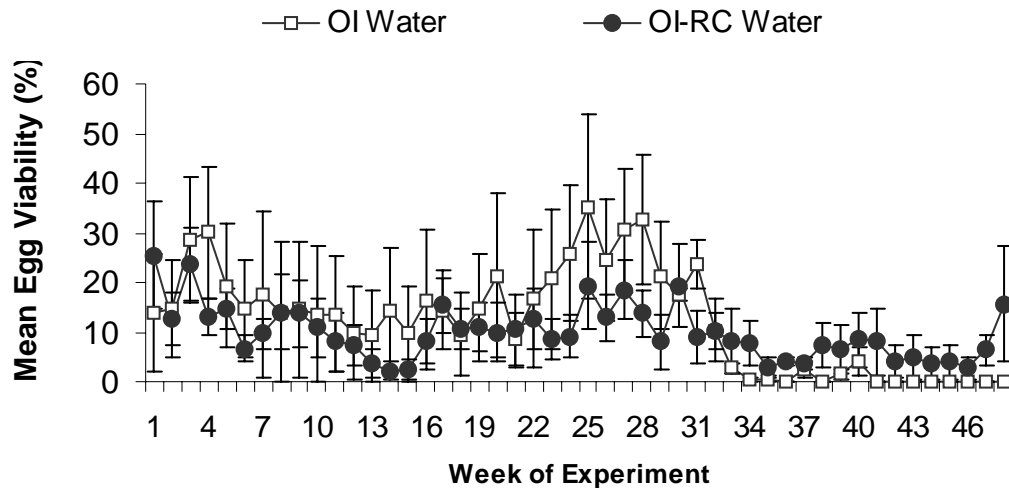


Figure 4.14. Flame angelfish weekly mean egg viability in OI water treated by water recirculation (OI-RC) vs. untreated OI water (OIW). OI water was treated via continual passes through a biological filter, foam fractionator and ultraviolet sterilizer. Data are presented as weekly means (n=5) \pm standard error of the mean.

4.5. Discussion

The results from this experiment lend support to previous observations at OI where flame angelfish egg quality was dramatically improved by transitioning from OI well-water to sterilized ocean water. As observed previously, flame angelfish fecundity, egg fertilization rates, and egg viability were all significantly improved when pairs were held in sterilized ocean compared to pairs in either treated (OI-RC) or un-treated OI well-water (OIW).

After 25 weeks, effects on fecundity were apparent as OIW treatment pairs exhibited a sharp decline in egg production. These pairs eventually (around week 42) ceased spawning regularly. After week 43, when spawns were recorded in this treatment group, they were very small and of poor quality. In contrast, fecundity from OW treatment pairs increased over the duration of the experimental period, particularly following the final transition of chlorination protocols (week 25), resulting in significantly greater average spawn sizes of approximately 1200 eggs per day. Treatment of OI water by use of recirculation technologies, including biological filtration, ultraviolet sterilization and protein skimming resulted in more consistent and slightly improved fecundity in OI-RC pairs compared to pairs in the untreated OI water (OIW) at the end of the experimental period. Pairs in the OI-RC treatment group spawned consistently throughout the trial, producing larger spawns (mean size of ~700 eggs per day) than OIW pairs. However, fecundity among those pairs was significantly lower at the end of the trial than pairs in the ocean water treatment.

As shown in the past at OI, flame angelfish produced large numbers of fertile and viable eggs in OI well-water for several months. However, as these data revealed, OI

water did not support sustained reproductive output in this species. Fertilization rates and egg viability eventually decreased significantly among pairs held in OI water (OIW) and were only marginally improved among the pairs in the OI water treated by recirculation (OI-RC). However, once chlorination protocols were rectified, pairs held in sterilized ocean water continued to produce spawns with average fertilization rates of 60%, similar to levels observed in 2005 at OI following their initial use of sterilized ocean water.

It remains unclear as to what characteristic of the OI water is responsible for the observed negative affects on flame angelfish egg quality. It also remains unknown why the use of OI water appears adequate for other tropical marine species at OI such as Pacific threadfin (*Polydactylus sexfilis*) and amberjack (*Seriola rivoliana*), which have remained in reproductive condition for several (more than 8 years for Pacific threadfin) years at OI. Well-water at OI exhibits low pH, elevated total gas pressure, and high CO₂ levels, which are typical characteristics of saltwater wells in this region. This well-water also mixes with some ground water as the salinity fluctuates from 31-33ppt, whereas the ocean water the well draws is nearly constant at 35ppt. Previous rigorous testing of OI water for elemental analyses revealed no recorded toxins resultant of ground water pollution, but did reveal a deficiency in available iodine (Laidely, et al. unpub.).

Low pH, elevated gas pressure, high CO₂ and reduced salinity levels can all be overcome through the use of recirculating systems as demonstrated in this trial. However, these parameters do not appear to be the primary causes of reduced egg quality in flame angelfish as correction of these parameters through water re-use only resulted in moderate improvement in egg quality. Reduced available iodine could affect egg quality in flame angelfish as iodine is known to be critical in a number of developmental and

reproductive functions. Therefore, further investigation into the role reduced iodine availability may be playing in the reduction of egg quality is warranted, as it might also be (more subtly) affecting the other species of research interest at OI. However, it should be noted that daily supplementation of OI well-water with iodine did not appear to improve flame angelfish reproduction, or egg quality, as tested briefly (6 weeks) following this experiment.

Another important, yet unexpected, result of this experiment was the demonstrated effect of chlorination level on egg fertilization rates. It became clear that as the level of chlorination of system replacement water increased, fertility rates decreased. However, it still remains unclear as to the exact mechanism underlying this effect. It is possible that increased chlorination levels resulted in the production of toxic by-products such as chlorophenols, as reported by Van der Toorn (1987). As phenols are derived from algal pigments, it is reasonable to conclude their presence may have been elevated in natural seawater that had just been nutrient loaded by coastal run-off, due to record rainfall in the region. Other potential chlorination byproducts include the formation of bromines and halo-amines (Johnson, 1977). Seawater contains approximately 65mg/L bromide and the addition of chlorine oxidizes the bromide to bromine, leading to the formation of organo-bromine compounds (Jenner et al., 1997). While the toxicity of the oxidant (chlorine) rapidly decreases, the same is not necessarily true for the more stable by-products.

As the practice of using chlorination to disinfect drinking water is widespread, there is a large body of literature on the byproducts formed during this process. However, there is limited information on analogous byproducts formed during the chlorination of

seawater (Jenner et al., 1997). These findings are important as the use of chlorination as a means of sterilization of seawater is widely used to control the growth of bio-film on heat exchangers in power stations, disinfect ballast water, reduce bio-fouling of membranes in desalination and to prevent diseases in marine aquaculture facilities (Fabbricino and Korshin, 2005). Although residual chlorine can be effectively neutralized, the potential byproducts of chlorination cannot be easily measured or eliminated.

In summary, it has been shown that the use of sterilized ocean water is superior to use of OI well-water for studies with flame angelfish, as use of OI water results in negative effects on egg quality. It is also apparent that the level of chlorination of the water used impacts egg fertilization, and therefore should be carefully administered. Chlorination levels should be set at the minimum level and duration required to effectively prevent targeted pathogenic organisms. In this study, treatment of ocean water at 25ppm for 30 minutes appeared adequate to prevent the occurrence of pathogens, while allowing for excellent egg quality. However, chlorinating at higher levels, and/or for increased durations resulted in negative effects on flame angelfish egg production, fertilization rates and egg viability.

4.6. Acknowledgments

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Chapter 5

DESCRIPTION OF THE EFFECTS OF BROODSTOCK DIET ON EGG QUALITY PARAMETERS AND SUSTAINED SPAWNING CHARACTERISTICS IN FLAME ANGELFISH (*Centropyge loriculus*)

5.1. Abstract

A series of studies was conducted to determine the effects of broodstock diet on flame angelfish (*Centropyge loriculus*) reproduction as well as to collect baseline spawning performance (mean fecundity, egg fertilization, egg hatching and larval survival to yolk-exhaustion) and egg biochemistry data for this species. Flame angelfish broodstock pairs (n=4) were maintained on one of four treatment diets (formulated Carnivore, formulated Herbivore, commercial diet Spectrum™, or Raw diet (consisting of squid, shrimp, fish eggs, and algae) and spawning performance (fecundity, egg fertilization rates and egg viability) was monitored daily for seven months. At the conclusion of the experiment, all the pairs were switched onto a diet regimen consisting of two daily feedings each of the Raw and Spectrum™ diets. Results from the feeding experiment indicated that although all the diets supported similar mean fecundity over the duration of the experiment, fertilization rates and egg viability were significantly greater among pairs receiving the Raw diet. Furthermore, spawning frequency was highest among pairs that received the Raw diet, which allowed for significantly greater numbers of fertile and viable eggs to be produced from that treatment group. Egg composition was generally reflective of the maternal diet and fatty acid profiles of flame angelfish eggs appeared similar to reported values for other tropical species. Daily egg collection from 18 pairs over a 20-month period revealed that flame angelfish are capable of sustained

daily egg production of 1,000-1,500 eggs per female with monthly maxima occurring near the new moon. Given adequate broodstock diet and environmental conditions, mean fertilization rates of 60-80% can be expected with average hatch rates of 80%, although turbulence of the hatching environment significantly reduced larval survival. Flame angelfish survival to yolk-exhaustion (72h post-hatch) was approximately 80% when reared in static tanks (26-27°C).

5.2. Introduction

The expansion of the marine ornamental aquaculture industry is dependent on overcoming several key “bottlenecks” including lack of suitable prey for first-feeding larvae as well as limited availability of larvae for feeding trials (Owstrowski and Laidley, 2001; Holt, 2003; Laidley et al., 2004). Additionally, poor egg quality can contribute to reduced larval survival during the known “critical period” of transitioning from endogenous yolk-reserves to exogenous prey capture (Rainuzzo et al., 1997). Variability in hatchery performance during these early life history stages is therefore largely dependant on parental contributions to the eggs (Kerrigan, 1997; Mc Cormick, 2003). However, despite its fundamental importance in aquaculture, broodstock nutrition remains one of the least studied and least understood areas of fish nutrition (Izquierdo et al., 2001). Furthermore, in marine ornamental species, the effects of broodstock condition on the eggs and larvae produced have been largely uninvestigated.

It has been demonstrated that, under culture conditions, improvement in nutrition and feeding of broodstock has positive effects on egg quality and seed production in numerous fish species (reviewed by Izquierdo et al., 2001). However, elaboration of these

relationships has been limited, in part, by the difficulty in providing sufficient numbers of replicates for experimental conditions, a complication that results from the fact that most commercially cultured marine finfish species are large, requiring large tanks and exceptional resources. Furthermore, many of those species take several years to reach sexual maturity, have limited yearly spawning cycles of weeks to months, and are therefore very difficult and expensive to replicate under a controlled study. Therefore, the use of limited replicates, and often long study durations, in this field of research has led to the slow growth of our knowledge and understanding of the effects of nutrition on egg production and egg quality.

Consistent production of high-quality eggs remains a significant bottleneck to the commercial culture of many marine fish (Kjorsvik et al., 1990). In addition, determining the effects of broodstock nutrition on egg production and egg quality continues to represent a significant challenge to fish culturists. Marine angelfish of the genus *Centropyge* could serve as a valuable model to further our understanding of marine fish broodstock nutrition while facilitating the continued expansion of marine ornamental aquaculture. *Centropyge* species produce eggs and larvae that share numerous characteristics with many other highly-valued ornamental species (Hioki and Suzuki, 1987; Hioki et al., 1990; Baensch, 2003; Sakai et al., 2003; Baensch, 2006; Olivotto et al., 2006). Therefore, developing culture technologies for these species could aid the development of culture methods for many other coral reef species.

While being representative of most marine ornamental species, with regard to type and size of eggs and larvae produced, angelfish of the genus *Centropyge* also exhibit several unique characteristics that make them ideal for studying the effects of parental

diet on egg quality. These characteristics include, but are not limited to the following: 1) small size and relative ease of husbandry; 2) early onset of sexual maturity (< 1 year); 3) sexual dimorphism; 4) widespread availability via the marine aquarium industry; and, 5) year round (daily) production of eggs. In particular, it is this last characteristic that sets these fish apart and lends these species so well to the study of broodstock nutrition.

Since comprehensive data on the spawning performance (sustained daily egg production), egg quality (egg fertility, egg viability, hatching rates and egg biochemistry) and early larval survival characteristics of captive flame angelfish have not been reported, the objectives of this study were to: 1) develop a thorough baseline data set of flame angelfish spawning performance consisting of egg production, egg quality characteristics and egg biochemistry from multiple spawning pairs; 2) formulate a diet that would be readily accepted by flame angelfish and capable of supporting sustained reproductive output; 3) investigate the effects of broodstock diet on spawning performance and egg quality in flame angelfish; and 4) determine the optimal environmental conditions for assessing egg hatching rates and for maintaining pre-feeding flame angelfish larvae to yolk-exhaustion.

5.3. Methods

5.3.1. Experiment Stocking and Environmental Conditions

These studies were carried out at the Oceanic Institute (OI) in Hawaii. Angelfish were obtained from a commercial supplier as individuals and paired according to external characteristics (see Chapter 2). Upon arrival at OI, fish were placed into 20L buckets and acclimated to the water conditions on site. After acclimation, fish were treated with a

2-minute freshwater dip to exclude any potential external parasites. Flame angelfish pairs were stocked (1 pair per tank) into 1,000L fiberglass tanks, which were all connected as part of the Outdoor Marine Ornamentals (OMO) recirculating system (Fig. 5.1). The tanks were kept bare, except for an artificial “reef” made out of PVC scaffolding, which served as hiding places for the fish. Lighting was provided by filtered natural sunlight, which penetrated the opaque plastic roofing material above the tanks. Temperature of the system was controlled by a heat pump, which maintained the water temperature at 26-27°C. Each week, approximately 10% of the system volume was replaced with new, sterilized ocean water (35ppt). Water quality parameters (temperature, salinity, DO, pH, NH_3 , NO_2 , and NO_3) were monitored weekly and additional water changes were performed if water quality deteriorated.



Figure 5.1. Photograph of a portion of the flame angelfish broodstock system at the Oceanic Institute. The system contained 19 identical, 1000L tanks that were configured into a recirculating system.

5.3.2. Broodstock Husbandry

5.3.2.1. Feeding. Broodstock pairs were fed one of four treatment diets, three to four times daily, to apparent satiation. The treatment diets consisted of a formulated Herbivore diet (pellets), a formulated Carnivore diet (pellets), a pellet commercial aquarium diet (Spectrum™ by New Life International, Inc.) and a Raw (frozen-wet) diet. The Herbivore and Carnivore diets were manufactured at OI facilities as described for other diets in Chapter 6. The composition of the two experimental formulated diets is provided in Table 5.1. These diets were purposefully formulated to be vastly different from one another, so as to determine whether the normally omnivorous angelfish would perform better on a diet formulated specifically for an herbivore or carnivore. The “Raw” diet (Table 5.2) was comprised of frozen squid, frozen shrimp, frozen fish eggs, frozen spinach, frozen peas, and dried algae (Nori). Raw diet ingredients were combined in a blender and the mixture was kept frozen until fed.

Table 5.1. Composition and proximate analyses of experimental diets formulated for flame angelfish and a commercially available aquarium diet.

Ingredient (% as fed)	Herbivore Diet	Carnivore Diet	Spectrum Diet
Fish Meal	15.00	53.40	N/A
Whole Wheat	16.62	6.72	N/A
Squid Meal	2.50	10.00	N/A
Brewer's Yeast	5.00	5.00	N/A
Soy Bean Meal	40.00	0.00	N/A
Vital Wheat Gluten	6.00	6.00	N/A
Krill Hydrolysate	3.00	2.00	N/A
Spirulina ¹	4.00	0.00	N/A
Magnesium Di-phosphate	0.56	0.56	N/A
Calcium Mono-phosphate	0.56	0.56	N/A
Sodium Mono-phosphate	0.56	0.56	N/A
Choline Chloride (Roche)	0.12	0.12	N/A
Mineral Mix (Cal Liquid) ²	0.12	0.12	N/A
Soy Lecithin (Central Soya)	3.00	3.00	N/A
Menhaden Oil	2.00	11.00	N/A
Vitamin C ³	0.16	0.16	N/A
Vitamin Premix ⁴	0.80	0.80	N/A
Proximate Analysis:			
Protein	44.30%	50.00%	37.60%
Lipid	8.40%	17.80%	9.59%
Moisture	8.60%	8.36%	8.74%
Ash	8.35%	10.29%	10.23%

¹ Spirulina Pacifica® by Cyanotech Corp., Kona, Hawaii

² Mineral mix contained CuSO₄*5H₂O (5.0mg Kg⁻¹), FeCl₃*6H₂O (72mg Kg⁻¹), MnSO₄*H₂O (24mg Kg⁻¹), KI (3.6mg Kg⁻¹), Se (0.25mg Kg⁻¹), and ZnSO₄*7H₂O (100mg Kg⁻¹). CoCl₂*6H₂O (1.2mg Kg⁻¹), and MoNa₂O₄*2H₂O (0.2mg Kg⁻¹)

³ Stay-C® (35% Vit.C) by Hoffman La-Roche, Nutley, New Jersey

⁴ Vitamin pre-mix (LV99.1) manufactured by Roche for OI included: Vitamin A (6000 IU Kg⁻¹), Vitamin D (2000 IU Kg⁻¹), Vitamin E (250 mg Kg⁻¹), Thiamin (40mg Kg⁻¹), Riboflavin (60mg Kg⁻¹), Pyridoxine (60mg Kg⁻¹), D-biotin (0.6mg Kg⁻¹), Niacin (80mg Kg⁻¹), Ca-Panthothenic Acid (180mg Kg⁻¹), Vitamin B₁₂ (0.096mg Kg⁻¹), Folic Acid (6mg Kg⁻¹), Astaxanthin (60mg Kg⁻¹), Menadione (40mg Kg⁻¹), Inositol (400mg Kg⁻¹)

Table 5.2. Composition of “Raw” control diet used for marine ornamentals at the Oceanic Institute¹.

Ingredient	% of Diet
Squid	27.71
Shrimp	27.71
Fish Eggs	18.48
Spinach	11.55
Peas	11.55
Nori	3.00
Proximate Analysis	
(As fed)	
Protein	7.97
Lipid	1.00
Moisture	89.37
Ash	1.35
(Dry weight basis)	
Protein	69.52
Lipid	11.07
Ash	1.35

¹ All ingredients were stored frozen. The squid, peas, spinach and nori were purchased from local supermarkets. Shrimp were provided by the OI shrimp department and the fish eggs were obtained from OI broodstock Pacific threadfin (*Polydactylus sexfilis*) or bluefin trevally (*Caranx melampygus*) surplus spawns.

5.3.2.2. Weight and Length Measurements. Flame angelfish were weighed and measured prior to, and at the conclusion of, the experimental period. Individual fish were removed from their tanks with a net, briefly dried with a soft towel, and then measured (total length) to the nearest millimeter by laying them over a table-mounted ruler. Fish were then transferred to a beaker of saltwater (pre-weighed) and individual weight measurements were recorded (± 0.1 g) using a bench-top scale.

5.3.2.3. Tank Maintenance. The tanks were routinely siphoned to remove any excess feed or debris and the tank walls and bottoms were scrubbed with a soft pad on a long pole once per week. Additionally, the PVC scaffolding, (Fig. 5.2) which served as an artificial “reef” for the fish, was removed once per week and cleaned thoroughly. Approximately twice per month, the fish were removed from their tank and moved to a clean tank, purposefully kept empty to serve as a rotation tank. Following being moved to the clean tank, the old tank would then be scrubbed, rinsed with a diluted bleach (2%) solution, rinsed with freshwater, dried, and then refilled to accommodate another pair.

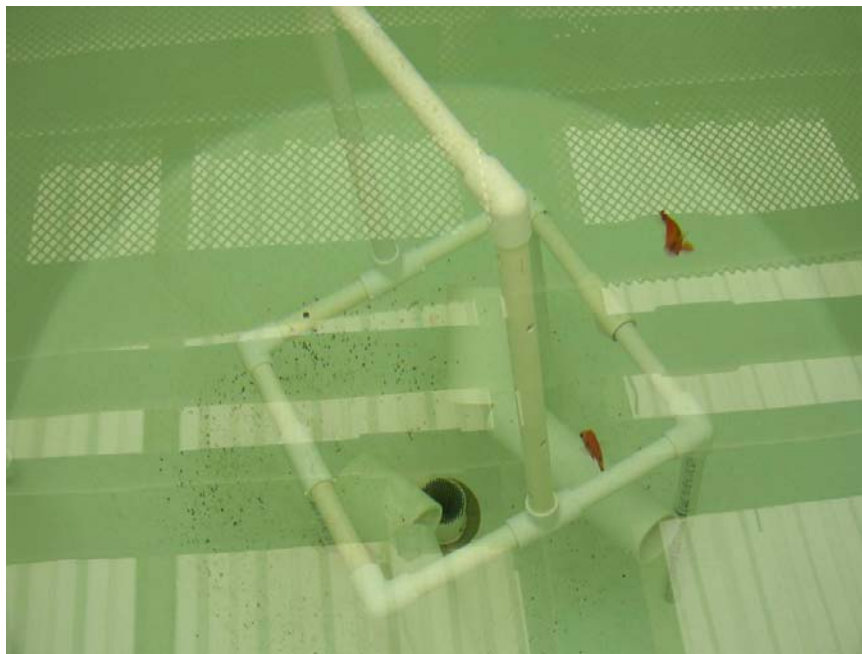


Figure 5.2. Photograph of flame angelfish near the PVC scaffolding used as an artificial “reef.” The structure was constructed to span from the bottom of the tank to come within reach of the surface. The frame was made from ½-inch PVC pipe, which supported numerous 2-inch PVC pipes for hiding places.

5.3.2.4. Egg Collection and Sampling. Eggs were collected daily using tank-mounted egg collectors (Fig. 5.3). Effluent water was drained from a surface drain through the egg collectors, which were equipped with a removable 200 μ m screen. The screen was slid down into position at the end of each day to concentrate eggs in the collector. Each morning, all of the collectors were emptied by opening the drain valve on the bottom into individual, clean 1L beakers (filled to approximately 800ml with clean seawater). The screens were then rinsed and left off the collectors throughout the day, so as to not collect waste and debris on the screens. If eggs were present, the beaker was placed under moderate aeration for a few minutes to homogeneously mix the eggs. While under aeration, five separate 10ml samples were taken and combined. Once all the samples were collected, they were poured into wells of a 12-well counting tray. The eggs were then counted under a dissecting scope and characterized as: 1) infertile (no clear cell division or other evidence of fertilization); 2) fertile-inviable (clear cell division but abnormally developed or development had arrested); or 3) viable (containing a fully developed embryo, ready to hatch). This procedure was repeated for each sample taken. Images of representative eggs in each of the above categories are presented in Figure 5.4.

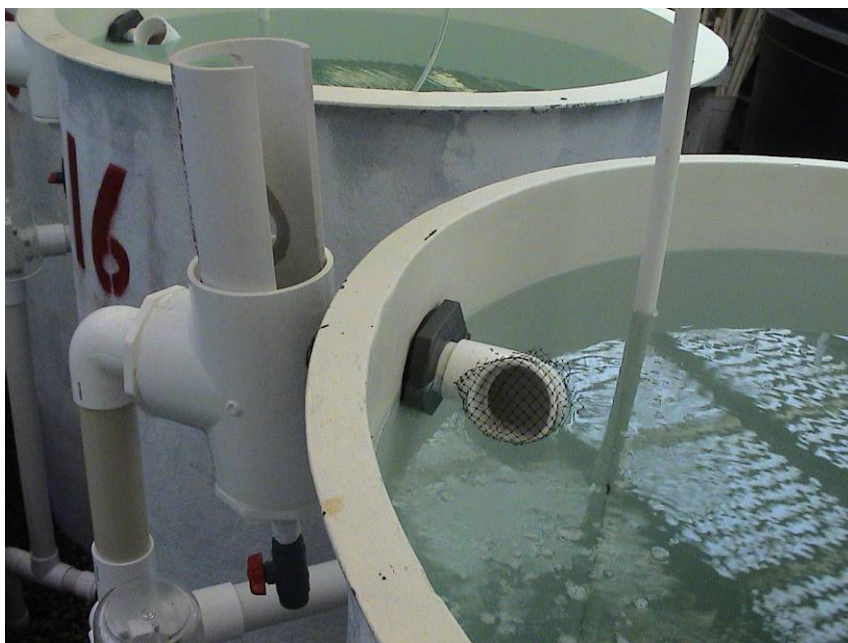


Figure 5.3. Photographs of tank surface drain emptying into egg collector (top) and close-up of water entering egg collector with screen up (bottom).

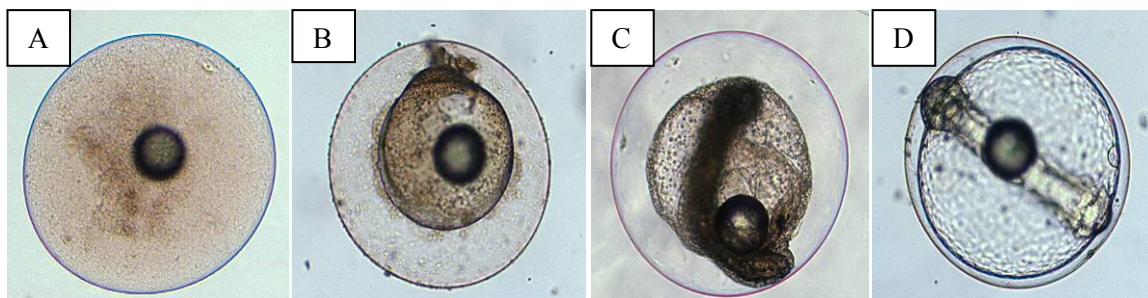


Figure 5.4. Photographs of representative egg quality categories for flame angelfish eggs. (A) Unfertilized egg (B) Fertile-inviable egg (arrested early) (C) Fertile-inviable egg (arrested late) (D) Viable egg

5.3.3. Egg Fatty Acid Analysis

In June of 2005, after four months of feeding on the treatment diets, egg samples were collected from each spawning pair at OI for fatty acid analyses. Over the course of 10 days, eggs were collected each morning as previously described. Following mixing for sub-sampling, each 1L beaker of eggs was allowed to settle to separate buoyant eggs from sinking eggs and other debris. Buoyant eggs were removed by pipette from the surface of the water and transferred to 12ml amber, glass tubes. Once approximately 300-500 eggs were transferred, the remaining seawater was removed from the tubes and the samples were rinsed three times with distilled water. The distilled water was removed by pipette, leaving < 1ml of water covering the eggs. The egg samples were then stored under nitrogen headspace in a -80°C freezer.

Egg samples were sent on dry ice to the Center of Marine Biotechnology (COMB), University of Maryland Biotechnology, Baltimore, Maryland where they were analyzed for fatty acid composition. All egg samples were freeze-dried, weighed ($\pm 0.1\text{mg}$), and ground. Lipids were extracted using a modified version of Folch et al. (1957) as described in Jackson and Place (1990). Samples were homogenized with 3.0ml

dichloromethane: methanol (2:1). The lipid extract was first washed with 0.88% potassium chloride water solution and then with dichloromethane: methanol: water (3:48:47). The extracts were then dried under nitrogen, weighed (± 0.1 mg), resuspended in 500 μ l of 1:1 methylene chloride: methanol, and capped under nitrogen.

Quantification of fatty acid methyl esters was achieved by hydrolyzing the extracted lipid with methanolic HCl and extracting the methyl esters into methylene chloride. An aliquot of the methylene chloride extract was subjected to gas chromatography directly on a Hewlett-Packard model 5980A instrument equipped with a flame ionization detector using a J&W DBWAX fused silica capillary column (30m x 0.25mm i.d., J&W. Scientific Inc., Folsom Ca.). Peaks were identified and quantified by comparison with retention times of known standards and expressed as percentages of fatty acid methyl esters.

5.3.4. Determination of Optimal Environment for Assessing Day 3 Survival

Expected survival of flame angelfish to day 3 (yolk exhaustion) was determined by placing up to 40 viable, buoyant eggs (from pairs receiving the Raw diet) per liter into 25L tanks (n=4). Eggs were volumetrically counted into the tanks containing seawater from the system the eggs came from. Each tank was brought up to exactly 25 liters and placed into a 26.5°C water bath. The tanks were exposed to a 12h L:12h D photoperiod and subjected to two treatments to test the effects of turbulence on survival. Tanks were either left static (no aeration) or lightly aerated for the 72-hour duration. No water exchange was provided for either treatment for the 72-hour experiment duration. Approximately 100,000 cells/ml of microalgae (*T-Isochrysis galbana*) were added to

each tank upon stocking to help maintain water quality. On the morning of day 3 post-hatch, the remaining larvae were counted and percent survival was determined.

5.3.5. Statistical Analysis

All data were analyzed using SYSTAT™ (ver 11.0). Normality of the data (Shapiro and Wilk, 1965) and homogeneity of the variance (Snedecor and Cochran, 1993) were tested. Percent data were transformed (arcsine) before conducting analysis of variance. Tukey's HSD test (Snedecor and Cochran, 1993) was used to determine significant differences among the means ($p < 0.05$).

5.4. Results

5.4.1. Flame Angelfish Size at Maturity

The length and weight distribution of male and female flame angelfish used in the diet experiments of this dissertation are presented in Figure 5.5. While males of this species were generally larger than females, the observed sizes for sexually mature individuals used in the current experiments were smaller than those reported for other species in this genus (Thresher, 1984). Significant growth was not observed over the course of this study. Sexually mature female flame angelfish ranged in size (length, weight) from 5.9cm, 4.8g to 7.5cm, 9.8g. The mean size for female individuals under this observation period was 6.58 ± 0.53 cm, 7.61 ± 1.67 g. Sexually mature male flame angelfish ranged in size from 7.2cm, 8.6g to 9.3cm, 17.8g. The mean size for male individuals under this observation period was 7.82 ± 0.63 cm, 11.43 ± 2.67 g.

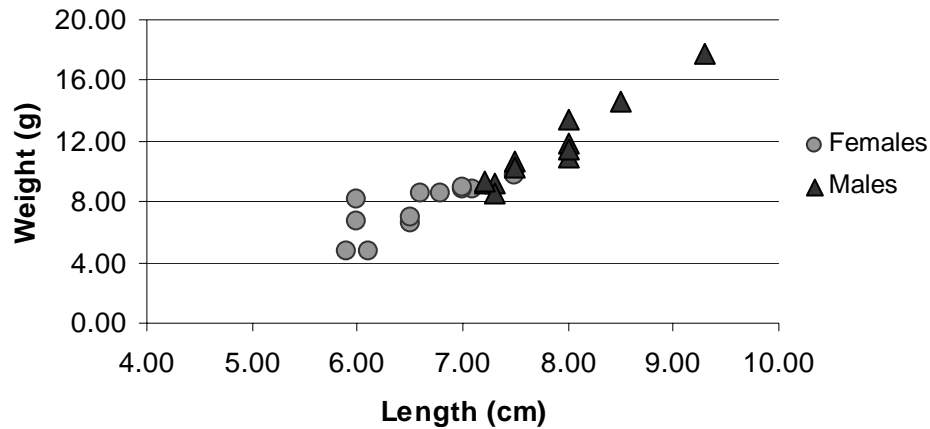


Figure 5.5. Length (TL) and weight of sexually mature male and female flame angelfish (n=36 individuals).

5.4.2. Effects of Diet on Flame Angelfish Egg Production and Egg Quality

Flame angelfish were fed their respective treatment diets from March through September 2005. Daily egg collection data revealed that as pairs were becoming reconditioned to the system, spawning was sporadic. Consistent daily egg production from all pairs was not achieved until near the conclusion of the experiment. Therefore, daily mean egg production, mean egg fertility rates and mean egg viability rates were calculated for each pair over the duration of the entire feeding experiment, rather than for each month. Treatment means were then calculated based on the individual pair means. Mean spawn size (Fig. 5.6) was approximately 500 eggs per day for pairs that received the Raw, Spectrum and Carnivore Diets. The pairs that received the Herbivore diet exhibited smaller mean spawn size (approximately 300 eggs) over the duration of the experiment, but this result was not significantly different ($p>0.05$) from the other treatments.

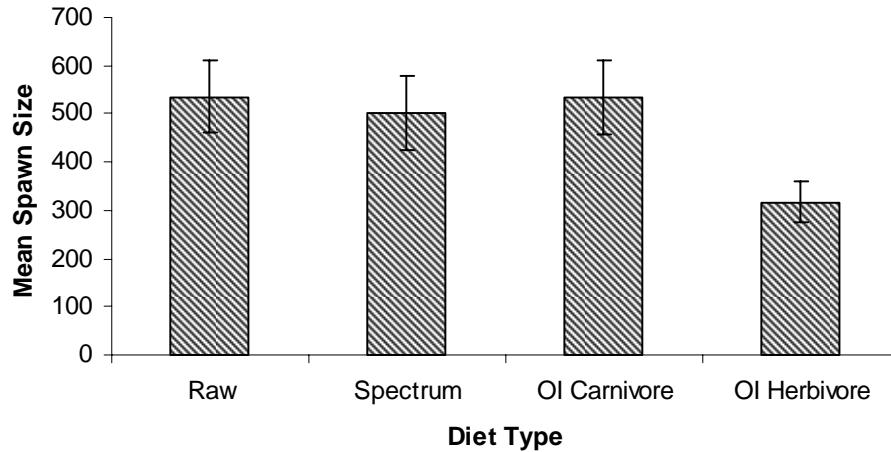


Figure 5.6. Flame angelfish mean daily spawn size on different diets over the duration of the 7-month feeding experiment (n=4 pairs). Data are presented as means \pm standard error.

Flame angelfish that received the Raw diet exhibited the highest fertility and egg viability of all the diet treatments (Fig. 5.7). Over the duration of the experiment, Raw diet treatment pairs maintained fertilization rates of approximately 65%. Pairs that received the Spectrum diet exhibited slightly, but not significantly, lower mean fertilization rates of 54%. Angelfish pairs that received the Carnivore and Herbivore diets had significantly lower mean fertilization rates over the duration of the experiment of 40% and 30% respectively. Mean egg viability was significantly higher among pairs that received the Raw diet (approximately 50%) than any of the other diet treatments. Pairs that received the Spectrum diet exhibited slightly, but not significantly, higher egg viability than pairs that received either the Carnivore or Herbivore diets.

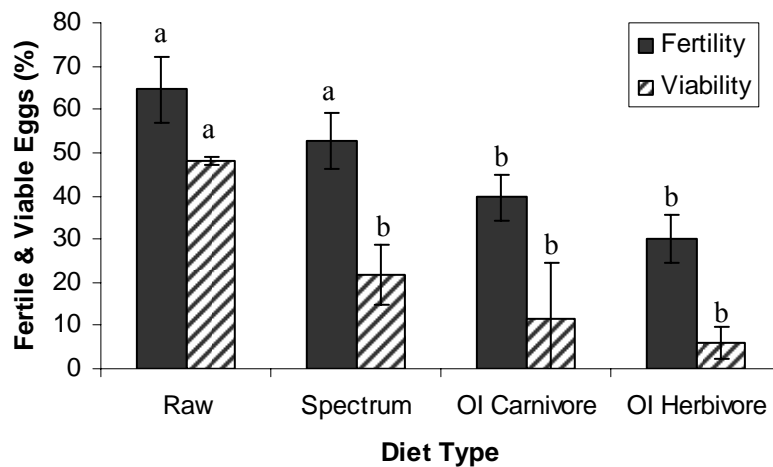


Figure 5.7. Flame angelfish mean egg fertilization and egg viability rates on different diets over the duration of the 7-month feeding experiment (n=4 pairs). Data are presented as means \pm standard error. Means with the same superscript are not significantly different ($p>0.05$).

At the conclusion of the seven-month feeding trial, pairs that received the Raw diet exhibited much better spawning performance (consistent egg production with high egg quality) than the pairs fed any of the formulated diets (Fig. 5.8). Although mean daily spawn size from pairs fed the Raw diet was not significantly greater than that of pairs fed the formulated diets (Fig. 5.6), mean monthly egg production and mean monthly viable egg production were much higher among pairs that were fed the Raw diet (Fig. 5.8). Increased spawning frequency (number of spawns per month) from pairs that received the Raw diet led to the over-all increased total egg production among those pairs.

In the last month of the experiment, mean monthly egg production from pairs fed the Raw diet was approximately 19,000 eggs per month, of which nearly all (97%) were fertile and approximately 50% were viable. Pairs that received the commercial Spectrum™ diet performed better than pairs on either of the OI formulated diets. Mean

monthly egg production was approximately 15,000 eggs per month, of which about 65% were fertile and 20% were viable. Pairs that received the OI Herbivore and OI Carnivore diets produced approximately 10,000 eggs per month. Neither of the latter treatment groups produced significant numbers of viable eggs; however fertility rates increased towards the end of the experiment and were approximately 60% and 48% in the Herbivore and Carnivore treatments, respectively.

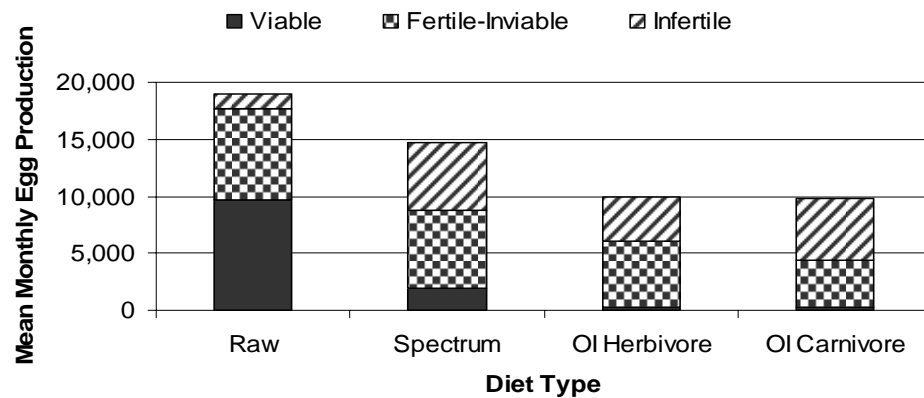


Figure 5.8. Flame angelfish mean monthly egg production for September 2005 at the conclusion of 7-month diet trial (n=4 pairs).

At the conclusion of the experiment all pairs were switched onto a “mixed” diet feeding regimen, which consisted of two daily feedings of the Raw diet and two daily feedings of commercial aquarium diets (Spectrum™ by New Life International, Inc and Bio-Blend™ by Marineland, Inc.). After one month of feeding on the “mixed” diet, egg production increased in all groups, except the group that previously received the Spectrum™ diet. This increase in egg production was attributed to increased energy intake, as pairs readily accepted the commercial aquarium diets compared to the OI formulated diets. However, viable and fertile egg production was still lower in the

treatment groups that were previously fed the OI formulated diets (Fig. 5.9).

Additionally, total fecundity was still significantly greater among pairs that had previously fed on the Raw diet. It was not until after four months of feeding on this “mixed” diet, that we observed any signs of the treatment groups recovering with regard to their spawning performance (Fig 5.10).

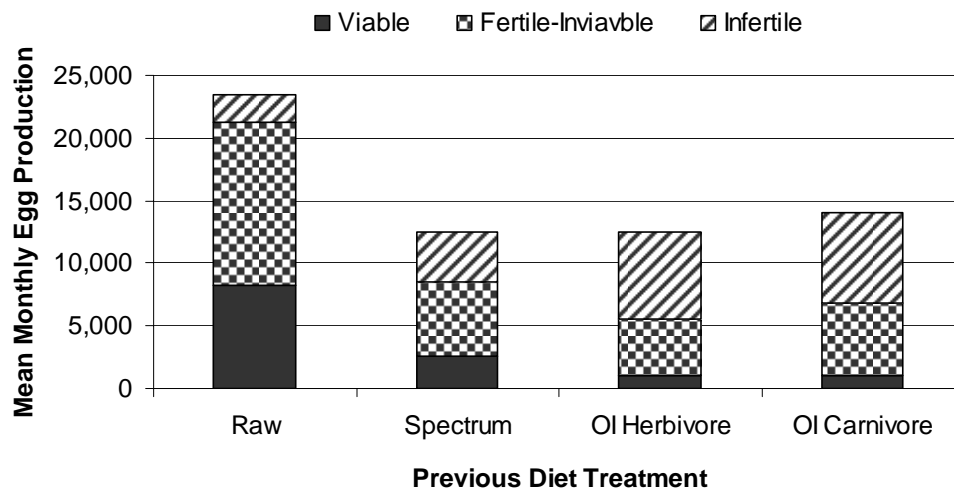


Figure 5.9. Flame angelfish mean monthly egg production after one month of feeding on a “mixed” diet (n=4 pairs).

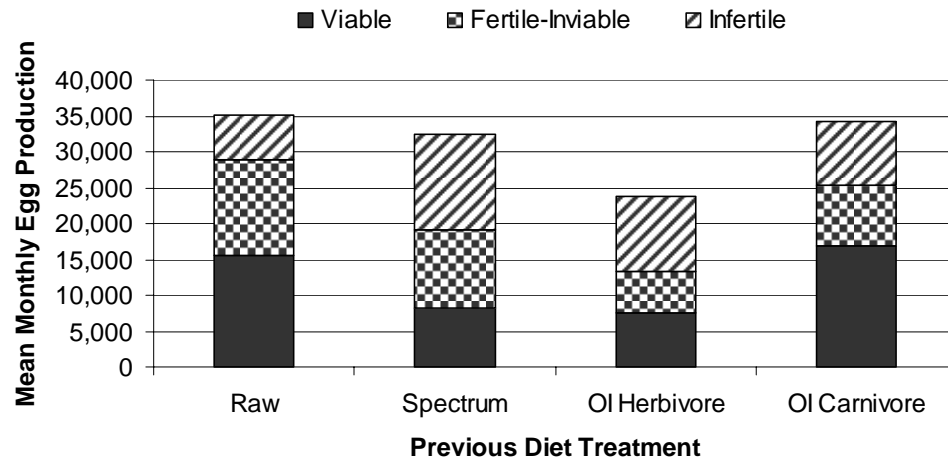


Figure 5.10. Flame angelfish mean monthly egg production after four months of feeding on a “mixed” diet (n=4 pairs).

5.4.3. Diet and Egg Biochemistry

Abbreviated results of the fatty acid analyses performed on the diets and egg samples from the different treatment groups are presented in Tables 5.3 and 5.4, respectively. Complete data from the fatty acid analyses of diets and eggs are presented in Appendix Tables A.1 and A.2 respectively. All of the formulated diets contained much lower levels of the polyunsaturated fatty acid (PUFA) linolenic acid (18:3n-3) as well as highly unsaturated fatty acids (HUFA) such as DHA (22:6n-3), EPA (20:5n-3) and ARA (20:4n-6) compared to the Raw diet. The Raw diet also exhibited the highest amount of n-3 HUFA (29.81% of total lipid), highest n-3:n-6 ratio (3.12) and highest DHA:EPA ratio (1.35) of all treatment diets. The OI formulated Herbivore diet contained the highest amounts of n-6 fatty acids, consisting predominately of linoleic acid (18:2n-6) originating from the large amount of soybean meal in that diet.

The composition of fatty acids in the eggs (Table 5.4) consisted mainly of 16:0, 16:1, 18:0, 18:1n-9, 18:2n-6, 20:4n-6 (ARA), 20:5n-3 (EPA), 22:5n-3 and 22:6n-3 (DHA) and generally reflected that of the maternal diets. However, levels of DHA and ARA appeared to have been preferentially sequestered in the eggs, as evidenced by their increased relative composition compared with the dietary source. DHA and EPA were highest in the eggs from fish fed the Raw diet. However, EPA values were lower in the eggs from all treatments than in their respective diets. DHA:EPA ratios in the eggs appeared to be somewhat conserved among treatments and were generally much higher in the eggs (1.16 to 3.14) than in the diets (0.72 to 1.35). EPA:ARA ratios were much lower in the eggs (0.56 to 2.84) than in the diets (7.77 to 11.49) and also appeared to be conserved among treatments with the exception of the Carnivore diet. Figure 5.11 illustrates the relationship between the fatty acid composition of the diets and the composition of the eggs.

Table 5.3. Fatty acid composition of experimental diets for flame angelfish broodstock. Values are reported as % of total fat.

Fatty Acid Profiles (%)

	Raw Diet	Spectrum Diet	OI Carnivore Diet	OI Herbivore Diet
14:0	1.97	6.50	6.15	3.64
16:0	24.75	23.61	25.38	20.94
16:1	1.91	5.57	7.76	4.71
18:0	6.64	4.41	5.56	4.69
18:1n-9	12.87	19.88	18.19	16.14
18:2n-6	9.43	15.16	11.82	30.68
18:3n-3	4.55	2.30	2.15	3.79
20:1	2.20	1.24	4.53	0.94
20:4n-6 (ARA)	1.59	0.85	0.58	0.41
20:5n-3 (EPA)	12.36	9.40	5.54	4.71
22:5n-3	0.77	0.89	1.16	1.00
22:6n-3 (DHA)	16.68	6.75	6.30	4.69
Others	4.32	3.44	4.90	3.32
Σ n-3	34.36	19.34	15.15	14.19
Σ n-6	11.02	16.01	12.40	31.09
n-3:n-6	3.12	1.21	1.22	0.46
Σ n-3 HUFA	29.81	17.04	13.00	10.40
DHA:EPA	1.35	0.72	1.14	1.00
EPA:ARA	7.77	11.06	9.55	11.49

Table 5.4. Mean fatty acid composition of total lipids in eggs from flame angelfish broodstock fed different diets. Values are reported as % of total fat.

Fatty Acid Profiles (%)

	Raw Diet¹	Spectrum Diet²	OI Carnivore Diet³	OI Herbivore Diet⁴
14:0	2.23 ± 0.06	2.44 ± 0.57	2.07 ± 0.48	1.60 ± 0.32
16:0	21.79 ± 0.79	17.45 ± 0.73	19.32 ± 1.87	15.87 ± 1.14
16:1	5.86 ± 0.04	6.55 ± 0.39	13.42 ± 2.24	4.29 ± 0.61
18:0	6.90 ± 0.27	7.64 ± 1.45	7.53 ± 0.43	7.50 ± 0.44
18:1n-9	10.15 ± 0.14	14.22 ± 3.94	10.78 ± 0.54	13.07 ± 0.80
18:2n-6	5.88 ± 1.03	8.64 ± 0.92	5.30 ± 0.50	19.01 ± 1.05
18:3n-3	0.89 ± 0.13	1.20 ± 0.08	0.99 ± 0.04	1.79 ± 0.13
20:1	0.68 ± 0.09	0.53 ± 0.20	0.65 ± 0.13	0.51 ± 0.03
20:4n-6 (ARA)	2.82 ± 0.41	2.87 ± 0.80	6.67 ± 1.64	1.29 ± 0.25
20:5n-3 (EPA)	7.72 ± 0.79	6.09 ± 0.13	3.52 ± 0.00	2.96 ± 0.34
22:5n-3	4.46 ± 0.12	4.51 ± 0.51	2.76 ± 0.02	2.96 ± 0.30
22:6n-3 (DHA)	17.36 ± 1.98	10.11 ± 1.16	6.85 ± 0.25	9.40 ± 1.45
Others	11.10 ± 0.18	14.37 ± 3.09	12.54 ± 0.21	12.51 ± 0.24
Σn-3	31.97 ± 1.26	24.89 ± 3.13	15.51 ± 0.15	18.68 ± 2.39
Σn-6	9.78 ± 1.47	12.68 ± 0.02	13.06 ± 1.24	21.93 ± 1.28
n-3:n-6	3.32 ± 0.37	1.96 ± 0.24	1.20 ± 0.13	0.84 ± 0.06
Σn-3 HUFA	30.17 ± 1.07	22.57 ± 2.39	13.83 ± 0.25	16.48 ± 2.17
DHA:EPA	2.30 ± 0.49	1.66 ± 0.16	1.95 ± 0.07	3.14 ± 0.15
EPA:ARA	2.84 ± 0.70	2.28 ± 0.59	0.56 ± 0.14	2.38 ± 0.24

¹ Calculated from six egg samples (n=2 pairs)

² Calculated from six egg samples (n=2 pairs)

³ Calculated from five egg samples (n=2 pairs)

⁴ Calculated from three egg samples (n=1 pair)

All results reported as means ± S.E.M.

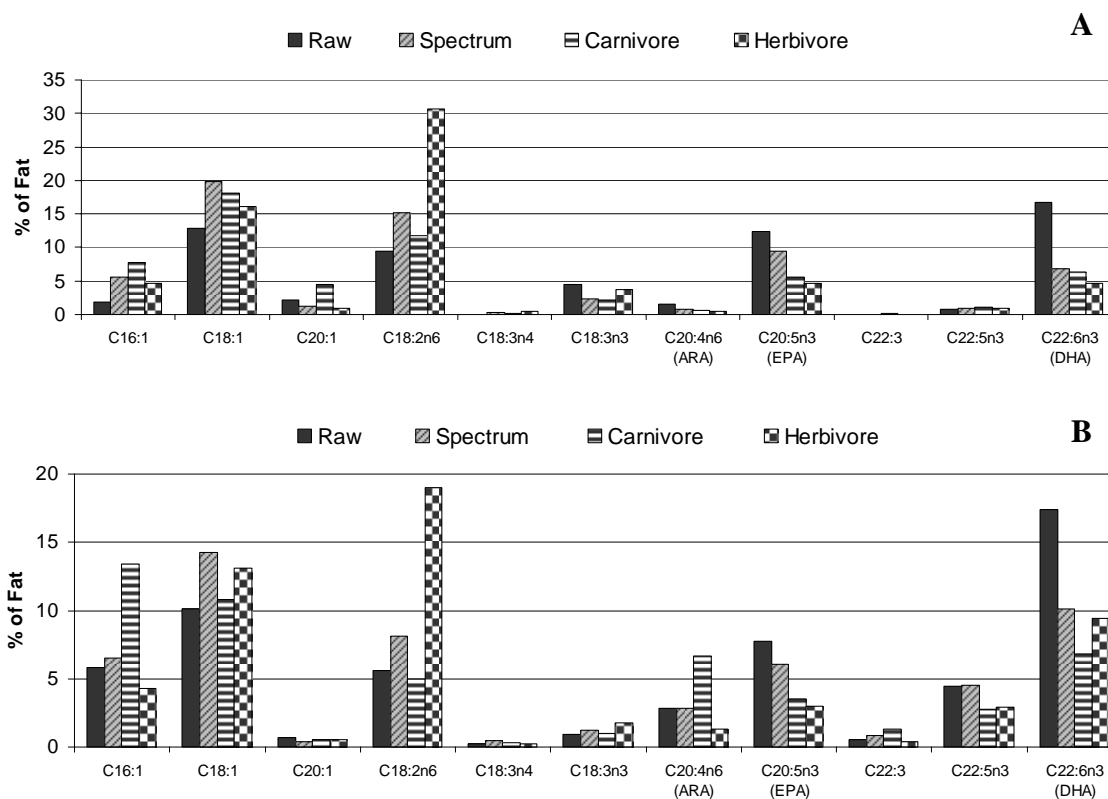


Figure 5.11. Relative fatty acid profiles of flame angelfish diets (A) and viable (buoyant) eggs (B) during feeding experiment. Diet samples n=1. Egg samples: Raw diet n=2 pairs; Spectrum diet n=2 pairs; Carnivore diet n=2 pairs; Herbivore diet n=1 pair.

5.4.4. Flame Angelfish Daily Egg Production

Flame angelfish mean daily egg production data from October 2005 through May 2007 are presented in Figure 5.12. Following the conclusion of the first diet experiment in September 2005, and subsequent change to the “mixed” feeding regime, mean egg production increased steadily from approximately 500 eggs per spawn to nearly 1,000 eggs per spawn. Peak, mean daily egg production then slowly decreased from April 2006 to January 2007, as pairs were subjected to additional diet experiments over that period (See Chapter 6). However, pairs continued to spawn consistently, averaging approximately 1,000 eggs per spawn. Following a transition back onto the “mixed”

feeding regime, in February 2007, egg production sharply increased to nearly 1,500 eggs per spawn. Although total egg production varied over this duration, monthly periodicity was consistently observed and appeared to correspond with the phases of the lunar cycle, with monthly maxima occurring at the dark phase of the moon.

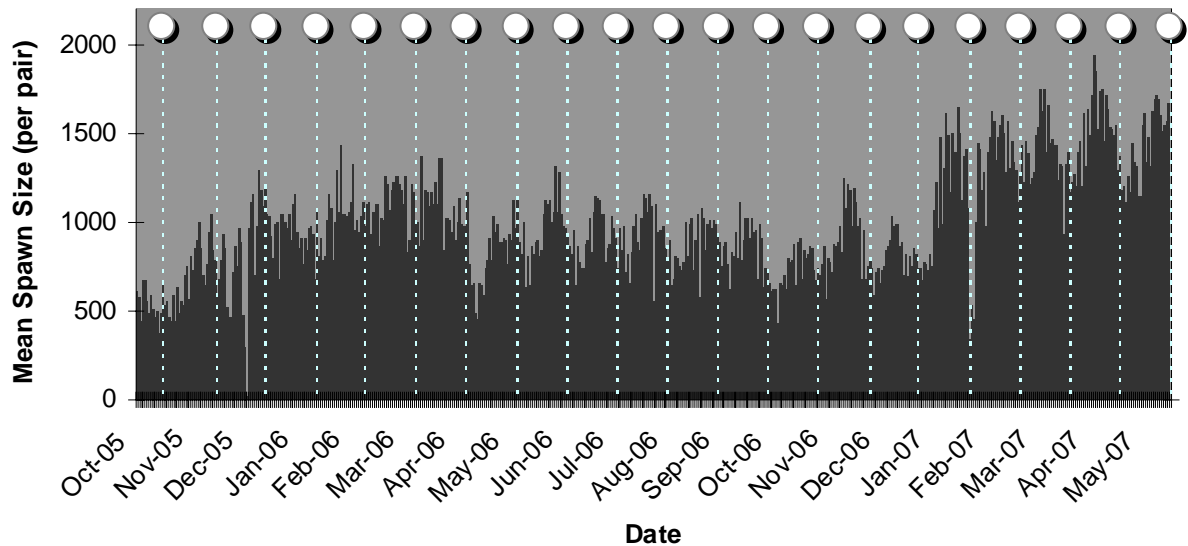


Figure 5.12. Flame angelfish mean daily egg production from October 2005 through May 2007 (n=18 pairs). Solid white circles indicate the dates of the full moon. Transition to “mixed” feeding regime began in October 2005. Subsequent diet experiments (Chapter 6) ran from April 2006 to January 2007. Return to the “mixed” feeding regime occurred late in January 2007.

5.4.5. Flame Angelfish Egg Hatch Rate and Larval Survival to Yolk-Exhaustion

The effects of turbulence on flame angelfish egg hatching and larval survival are illustrated in Figure 5.13. Hatch rates were lower (not significant at $p > 0.05$) among aerated tanks, with mean hatch rates of 64% compared to non-aerated tanks (mean 83%). However, survival of flame angelfish yolk-sac larvae was significantly reduced in aerated tanks. Flame angelfish mean survival to 72h within aerated tanks was $< 5\%$. Mean survival to 72h in non-aerated tanks was approximately 79%.

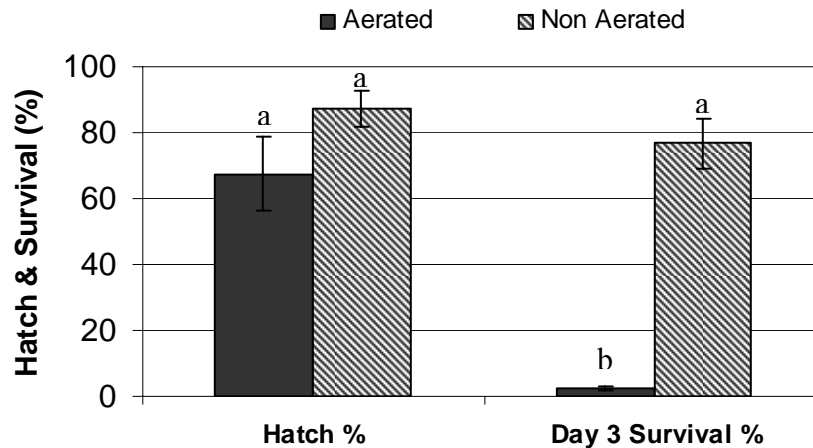


Figure 5.13. Flame angelfish mean egg hatching and survival of pre-feeding larvae to 72h in aerated vs. non-aerated 25l culture tanks (n=4 tanks). Data are presented means \pm standard error. Means with the same superscript are not significantly different ($p>0.05$).

5.5. Discussion

The results of the feeding experiment demonstrated that flame angelfish could be conditioned to spawn on single, formulated diets for prolonged time periods (> 6 months) and that egg production, egg quality and egg composition were significantly affected by the broodstock diet. Although moderate egg output with low fertility rates was achieved on all of the formulated diets tested, total fecundity and over-all egg quality on the formulated diets were not comparable to the Raw diet. Flame angelfish pairs that received the Raw diet exhibited higher egg fertilization rates and egg viability rates than pairs on any of the formulated diet treatments (Fig. 5.7). Furthermore, flame angelfish pairs that were fed the Raw diet spawned more consistently throughout the experimental period, which allowed for greater total monthly egg production in that treatment (Fig. 5.8). The commercial aquarium diet, Spectrum™, supported more consistent spawning that resulted in improved egg production and greater egg viability compared to OI formulated

diets. However, despite relatively high egg fertility rates (65%), egg viability rates among pairs that received the Spectrum™ diet remained lower than in pairs that were fed the Raw diet.

Despite large differences in dietary ingredients, the OI formulated diets appeared to elicit similar, negative characteristics in angelfish egg production and egg quality. In general, pairs receiving these diets spawned less consistently and produced fewer eggs per spawn than pairs receiving the Spectrum™ and Raw diets. Furthermore, egg quality among pairs in both of the OI diet treatments was significantly reduced compared to pairs that were fed the Raw diet. However, egg quality characteristics (egg fertility and egg viability) were not significantly different between the two OI diet treatments.

Following the experimental feeding period, all the flame angelfish pairs were switched to a “mixed” feeding regime, consisting of the Raw diet plus commercial aquarium diets, that resulted in improved egg production and egg quality in all pairs, regardless of previous treatment. Interestingly, pairs that were previously fed the Raw diet also exhibited increased fecundity and egg quality as a result of this changed feeding regimen. This indicated that despite supporting the observed excellent spawning characteristics during the preceding experiment, the Raw diet may have been limiting in some essential nutrients, or over-all dietary energy. The resulting increase in egg production and egg quality revealed that flame angelfish were capable of exhibiting higher fecundity and better egg quality than reported during the feeding experiment. Therefore, future flame angelfish “Control” diets should incorporate a mixture of raw ingredients and commercial diets as these dietary components appear complimentary and this feeding regimen has yielded the best spawning performance to date.

In examination of the fatty acid analyses of the diets, it was interesting to observe that the Raw diet had higher levels of n-3 HUFA than any of the formulated feeds. In particular, DHA and EPA were highest in the Raw diet, followed by the Spectrum™ diet. Deficiency in the n-3 HUFA, particularly DHA and EPA, has been linked to reduced fecundity, and decreased fertilization, hatching and viability of marine fish eggs (Rainuzzo et al., 1997). ARA was also highest in the Raw diet and was nearly two fold higher than in any of the formulated diets. In studies with European sea bass (*Dicentrarchus labrax*) broodstock, individuals fed an increased level of ARA produced significantly better quality eggs (Bell and Sargent, 2003). In the current experiment, total fecundity and egg quality (fertilization and viability) were lower in the groups of fish receiving the diets with the lower n-3 HUFA and ARA levels. However, all the tested diets were dramatically different in their composition, so direct comparison cannot be definitive.

Collection of eggs from multiple flame angelfish pairs allowed for the first documentation of fatty acid profiles in eggs from this species (Table 5.4; Appendix B). Flame angelfish egg fatty acid composition was closely related to parental diet composition as reported with other species of marine fish (Li et al., 2005). Generally, n-3 HUFA (DHA and EPA) levels were lower in flame angelfish eggs than for temperate species such as halibut (*Hippoglossus hippoglossus*) (Cameron, 2006) or Japanese flounder (*Paralichthys olivaceus*) (Furuita et al., 2003) and were more similar to levels reported for tropical species such as striped jack (*Pseudocaranx dentex*) (Vassallo-Agius et al., 2001) or crescent sweetlips (*Plectrolynchus cinctus*) (Li, et al., 2005). However, observed ARA levels in flame angelfish eggs were much higher than values reported for

other marine species (Bruce et al., 1999; Vassallo-Agius et al., 2001; Li, et al., 2005; Cameron, 2006) and were only similar to eggs from temperate species which had been effected by deliberate, increased ARA content of the maternal diets (Furuita et al., 2003; Cameron, 2006). The observed elevated ARA content in flame angelfish eggs is similar to levels observed in other tropical species, such as mangrove red snapper (*Lutjanus argentimaculatus*) (Emata et al., 2003) and Pacific threadfin (*Polydactylus sexfilis*) (Laidley et al., unpublished data). Emata et al. (2003) suggested that ARA might be nutritionally more important for egg development and larval survival in tropical species, than in temperate species as evidenced by elevated egg ARA content relative to eggs from species such as cod, herring or sea bass. However, further study will be necessary to determine if egg ARA level is correlated with flame angelfish egg quality.

Following the feeding experiment, a comprehensive baseline dataset spanning over 20 months of daily egg production from 18 individual, spawning pairs was generated and provided significant insights as to the captive spawning characteristics that could be expected for this species. Spawning data collected from October 2005 through May 2007 demonstrated that flame angelfish are capable of sustained, daily egg production in the range of 1,000-1,500 eggs per day and that periodicity in their egg production was closely matched to the lunar cycle (Fig. 5.12). Lunar periodicity in reproductive cycles for species of *Centropyge* has, until now, been unconfirmed. Previous research by Bauer and Bauer (1981) and Laidley, et al. (2004) had suggested that *Centropyge* do not exhibit lunar periodicity in their spawning cycles. However, it is likely that the use of fewer replicates, even for prolonged time periods, had led those authors to those conclusions. Our data indicate that while flame angelfish can spawn daily for

protracted time periods (>2 years for the oldest pairs observed), maximum egg output can be observed near the middle (dark phase) of the lunar cycle.

In our daily evaluation of egg quality characteristics, egg viability was the most variable of all the parameters measured. Mean egg viability was generally 50% of all eggs produced, even with high (>90%) fertility rates. It was relatively common to observe 70-80% viable eggs one day and then 30-50% the next. Furthermore, egg viability varied widely day-to-day, within treatment groups, indicating that daily fluctuations in this parameter was not necessarily linked to treatment. There did not appear to be any clear patterns to explain this day-to-day variability and we assume this is either part of this species' natural egg production cycles, or an artifact of their captive condition. By utilizing the monthly mean (28-31 data points) of egg quality characteristics (fecundity, fertility and egg viability), variability was minimized within treatment groups and differences between treatments could be assessed.

Increased broodstock population size and improved broodstock husbandry and feeding led to an increased supply of viable eggs. However, high mortality of the pre-feeding larvae remained a considerable bottleneck (Laidley et al., 2004). Larviculture research with flame angelfish revealed that the larvae were far more delicate than many other cultured fish species reared at OI. Therefore, physical, environmental parameters affecting survival during the early, pre-feeding stages of development needed to be identified and overcome. Our results demonstrated that flame angelfish larvae could not tolerate even mild aeration, as survival of larvae to day 3 post-hatch in aerated tanks was less than 5%. This was a significant finding, as most marine fish culture protocols suggest

at least mild aeration of rearing tanks. However, by simply allowing the tank to remain static, high (80%) survival to yolk-exhaustion was achieved.

In summary, this study demonstrated that flame angelfish can be maintained, and conditioned to spawn on formulated diets, although reproductive output and egg quality was best among fish fed the Raw diet. Dietary factors such as n-3 HUFA and ARA level could have caused some of the observed treatment differences and are therefore worthy of future investigation. Despite being maintained on dramatically different diets for over seven months, flame angelfish displayed the ability to adapt to new diets quickly and broodstock pairs used in this diet experiment could potentially be used in future experiments.

Since flame angelfish displayed daily spawning it was originally anticipated that diet effects would be manifested quickly in observable egg characteristics. However, it was not until after four months of feeding on the “mixed” regime, that we detected any signs of the formulated diet treatment groups recovering with regard to their spawning performance. Therefore, due to the observed delay of improved egg quality characteristics in response to diet, it is suggested that future diet trials be planned for a minimum of four-month periods, with a time gap in between to allow pairs to recover before commencing another study.

In addition to egg quality characteristics, early larval developmental traits and survival to yolk-exhaustion are also affected by maternal diet. Therefore, flame angelfish larvae could be used to investigate the effects of broodstock diet on larval characteristics. Survival rates to yolk-exhaustion of approximately 80% can be expected with this species, given broodstock care and larval rearing conditions described herein. Due to

demonstrated ease of conditioning, adaptability to formulated diets, sustained reproductive output and simplicity of maintaining pre-feeding larvae, flame angelfish could serve as an excellent model for future studies on factors affecting marine fish egg and larval quality. Future studies should focus on development of formulated diets that support spawning performance, at least, equal to that of the described “mixed” feeding regimen so that individual dietary components can be then thoroughly evaluated.

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Chapter 6

STUDIES ON THE EFFECTS OF DIETARY n-3 HUFA LEVEL ON FLAME ANGELFISH (*Centropyge loriculus*) EGG AND LARVAL QUALITY

6.1. Abstract

A series of experiments was conducted to determine the effects of dietary n-3 highly unsaturated fatty acids (HUFA) on flame angelfish (*Centropyge loriculus*) egg and larval quality. In the first experiment, formulated diets containing 1.84, 2.97 or 3.63% n-3 HUFA were fed to flame angelfish broodstock (n=4) for 5 months. In the second experiment, formulated diets containing higher levels of n-3 HUFA (2.67, 3.48 or 4.34%) were fed to flame angelfish broodstock (n=4) for 5 weeks. Mean daily egg production, egg fertilization rates and egg viability were used as indicators of egg quality in both experiments. Additionally, in experiment 1, mean egg diameter, oil globule diameter, percent hatch, larval size at hatch, percent survival to yolk-exhaustion and larval size at yolk-exhaustion were recorded for each treatment. Flame angelfish that were fed the diet containing 3.63% n-3 HUFA exhibited significantly increased fecundity, fertilization rates and egg viability than fish that were fed the other two formulated diets. Furthermore, egg diameter, oil globule diameter, larval size at hatch, percent survival to yolk-exhaustion, and size at yolk-exhaustion from fish that were fed the diet containing 3.63% n-3 HUFA were not significantly different from that of fish fed the Control diet. In Experiment 2, egg quality significantly decreased among all fish that were fed the formulated diets whereas fish that were fed the Control diet exhibited relatively good egg quality over the same period. However, results from Experiment 2 revealed that flame angelfish egg quality could respond rapidly (within weeks) to maternal dietary changes

indicating that future diet experiments could be planned for shorter time periods than previously reported.

6.2. Introduction

It has been demonstrated that improvement in nutrition and feeding of broodstock has positive effects on egg quality and seed production in many marine fish species (reviewed by Izquierdo et al., 2001). Lipid and fatty acid composition of broodstock diets have been shown to affect both successful reproduction and larval survival in marine species such as Gilthead sea bream (*Sparus aurata*) (Fernandez-Palacios et al., 1995) and sea bass (*Dicentrarchus labrax*) (Bruce et al., 1999). The significance of lipids for developing marine fish larvae has been reviewed by Rainuzzo et al. (1997) and Sargent et al. (1999). Dietary lipids are sources of essential fatty acids needed for growth and survival. Marine fish larvae require highly unsaturated fatty acids (HUFA) of the n-3 series such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) due to their inability to synthesize them (Rainuzzo et al., 1997). These fatty acids are present in various species of phytoplankton and concentrated by grazing zooplankton, which the larvae ultimately consume as their primary food source. Recently, the role of arachidonic acid (ARA) of the n-6 series as another dietary essential fatty acid has been reviewed (Bell and Sargent, 2003). Those authors stressed that future work focus not only on the quantities of DHA, EPA and ARA present, but also on the ratios between them as being critical in further understanding their effects on development.

In marine aquaculture, there have been numerous studies linking DHA and EPA levels in broodstock diets to egg and larval quality (Fernandez-Palacios et al., 1995;

Bruce et al., 1999; Furuita et al., 2000 & 2002). Recently, dietary ARA has also been shown to affect egg and larval quality (Bell and Sargent, 2003; Furuita et al., 2003). Deficiency in the n-3 HUFA has been linked to reduced fecundity, and decreased fertilization, hatching and viability of marine fish eggs (Rainuzzo et al., 1997). In studies with European sea bass broodstock (*Dicentrarchus labrax*), individuals fed an increased level of ARA produced significantly better quality eggs (Bell and Sargent, 2003). However, similar studies with Japanese flounder (*Paralichthys olivaceus*) demonstrated that high levels of ARA had a negative effect on egg quality (Furuita et al., 2003), as did elevated levels of total n-3 HUFA (Furuita et al., 2002). Clearly, the dietary levels of DHA, EPA and ARA required for optimal egg and larval quality may be species-specific. However, their impact on egg and larval quality seems to be universal among marine species.

Although much work has been carried out on marine food fish species, little has been reported for coral reef fish with regard to broodstock nutrition and its effect on egg quality and/or larval survival. These effects are likely magnified in coral reef species, of which many exhibit continuous spawning strategies and short vitellogenic periods. However, this project would be the first to investigate such effects on this species. Conditioning broodstock for optimal production is the cornerstone of any hatchery operation. Therefore understanding the nutritional requirements of this species and how the adult diet impacts the eggs and larvae they produce are critical components worthy of further investigation.

The objectives of this series of studies were to do the following: 1) develop a formulated broodstock diet that is readily accepted by flame angelfish and supports

sustained spawning characteristics comparable to results achieved using “Raw” diets (Chapter 5); and 2) use the formulated diet in a series of experiments to examine the effects of the n-3 HUFA on flame angelfish egg and larval quality.

6.3. Methods

6.3.1. Experiment 1: Effects of Dietary n-3 HUFA Level on Flame Angelfish Egg and Larval Quality

6.3.1.1. Pair Ranking and Assignment to Treatment Groups. Following six months of feeding on a “mixed” diet regimen as described in Chapter 5, flame angelfish pairs (used in previous diet experiments) were ranked according to egg quality criteria. For each pair, means of the preceding three months’ data for egg viability, fertilization rates and fecundity were calculated. Pairs were then sorted by these three characteristics, respectively, into three blocks, as high, med or low spawning performance. A fourth block was added, as some new pairs from which we had no spawning data, were also included. Each of the four blocks contained four pairs, to which one of the four treatments was randomly assigned. In this way, each treatment would be randomly allocated to a pair from each of the three spawning performance blocks in addition to one new pair.

6.3.1.2. Experiment Stocking and Environmental Conditions. Flame angelfish pairs were stocked (1 pair per tank) into 1,000L fiberglass tanks, which were all connected as part of the Outdoor Marine Ornamental (OMO) re-circulating system (see Chapter 5). The tanks were kept bare, except for an artificial “reef” made out of PVC scaffolding, which served as hiding places for the fish. Lighting was provided by filtered natural sunlight, which penetrated the opaque plastic roofing material above the tanks.

Temperature of the system was controlled by a heat pump, which maintained the water temperature at 26-27°C. Each week, approximately 10% of the system volume was replaced with new, sterilized ocean water (35ppt). Water quality parameters (temperature, salinity, DO, pH, NH₃, NO₂, and NO₃) were monitored weekly and additional water changes were performed if water quality deteriorated.

6.3.1.3. Diet Formulation. The primary ingredients in the experimental diets were pollock meal and squid meal. These ingredients were included in approximately equal proportions, as 50% replacement of fish meal by squid meal was found to significantly increase egg quality in striped jack (*Pseudocaranx dentex*) (Vassallo-Agius et al., 2001a) and Yellowtail (*Seriola quinqueradiata*) (Vassallo-Agius et al., 2002).

The amino acid requirements for flame angelfish are currently unknown. Therefore the protein levels of the diets were set high (formulated at 60%), using high-quality protein sources, to maximize the chances of meeting all essential amino acid requirements.

The energy requirement for coral reef fish may be higher than for other marine species, as these fish spawn very frequently (some spawn daily). Therefore, the lipid content of the diets was also set higher (formulated at 16%) than that of commercial aquarium diets (6-10%).

The total n-3 HUFA levels of the experimental diets were formulated to range from 2.12 to 3.83% of diet dry weight. This range was higher than the recommended 1.5 to 2% recommended for sea bream (Fernandez-Palacios, et al., 1995) and Japanese flounder (Furuita et al., 2000) but, similar to the levels reported to be favorable for striped jack (Vassallo-Agius et al., 2001b and 2001c). Arachidonic acid was included from 0.11 to 0.24% of the diet dry weight, as Furuita et al. (2003) reported that inclusion of

arachidonic acid up to 0.6% of the dry weight improved egg quality in Japanese flounder. Moreover, Sargent et al. (1999) reported that both the concentration, as well as the ratio of all three essential HUFA (DHA, EPA and ARA), was important in marine larval fish nutrition. Therefore, a ratio of 10:5:1 (DHA:EPA:ARA) was targeted in these diet formulations as recommended by Sargent et al. (1999). The individual HUFA levels were manipulated by the inclusion of commercially available products, containing high levels (approximately 40%) of either DHA (DHAsco™ by Martek Biosciences Corp., Columbia, Maryland) or ARA (Vevodar™ by DSM Food Specialties Inc., Parsippany, New Jersey). Where necessary, fish oil was substituted with olive oil to reduce the n-3 HUFA level, while maintaining equal over-all lipid level.

Carotenoids, particularly astaxanthin, have been found to significantly affect egg quality, via egg production, and are important sources of vitamin A and antioxidants (Vassallo-Agius et al., 2001b and 2002). For this reason, spirulina and Naturose® (an algal source of astaxanthin) were included in the diet. Spirulina was chosen for its desirable composition of essential amino acids, vitamins, minerals and high concentration of carotenoids. Naturose® is a commercial algal product that contains 15,000ppm astaxanthin and is widely used in the salmon and shrimp farming industries. The manufacturer suggests inclusion levels in diets for trout and salmon at 45-90ppm. As formulated, the experimental diets contained 100ppm astaxanthin.

Vitamin C and vitamin E were added to the diet in addition to what was supplied in the vitamin pre-mix. This was due to the demonstrated importance of these two vitamins in reproduction and their reported effects on egg quality (Lee and Dabrowski, 2004 and Emata et al., 2000). Furthermore, the requirements for vitamin E could

be greater in species that require higher total fat (and HUFA levels); and therefore additional vitamin E was added to the diets to act as an in-vivo antioxidant.

6.3.1.4. Diet Processing. The experimental diets were individually manufactured at OI facilities as follows: The major dry feed ingredients with a particle size of less than 250 μm , were mixed for 15 min in a Hobart food mixer (Model D-300, Hobart Manufacturing Corporation, Troy, Ohio). A warm (approx. 60°C) aqueous solution of sodium phosphate, potassium phosphate, choline chloride, and trace element premix, was added to the dry ingredient mix to bring the moisture content of the resulting mash to approximately 25%. The resulting mash was blended for a further 15 min. Half the supplemental oils, lecithin, vitamin E, ethoxyquin, and Naturose® were blended in a KitchenAid mixer (Model K5SS, KitchenAid, St. Joseph, Michigan), and added to the mash and mixed for a further 15 min. The mash was then pelleted by a California Pellet Mill (Model CL5, San Francisco, California) fitted with a 1.8 mm diameter die. No steam was used and the pellet temperature at the die was below 70°C. The moist pellets were dried overnight in a drying cabinet using an air blower circulating ambient air until the moisture level was below 10%. Once dried, the pellets were then crumbled and then sieved to obtain the final desired pellet size of 1.5-1.8mm. These pellets were then top coated with the remaining ingredients. The vitamin premix and vitamin C source were emulsified with the remaining oils, vitamin E, ethoxyquin, lecithin and Naturose® in a KitchenAid mixer, and added to the dry cooled pellets by top coating using a Hobart D300 food mixer with a whisk beater. The finished pellets were stored in sealed plastic bags at -20°C until used.

6.3.1.5. Feeding. Flame angelfish broodstock pairs were fed one of four treatment diets to apparent satiation four times daily. The treatment diets consisted of a formulated low n-3 HUFA diet (Low n-3), a medium n-3 HUFA diet (Med n-3), a high n-3 HUFA diet (High n-3), and a Control diet. The Control diet was formulated and administered as described in Chapter 5 and consisted of a “mixed” feeding regime. This regime consisted of two daily feedings of the “Raw” diet and two daily feedings of a commercial aquarium diet (Spectrum™ by New Life International, Inc). The composition of the three experimental formulated diets are given in Table 6.1. The calculated proximate composition and HUFA content of the experimental formulated diets are provided in Table 6.2.

Table 6.1. Composition of formulated diets fed to flame angelfish broodstock in Experiment 1.

	Treatment Diet		
	High n-3	Med n-3	Low n-3
Ingredient:	(g/100g)	(g/100g)	(g/100g)
Pollock Meal	30.00	30.00	30.00
Squid Meal	35.00	35.00	35.00
Menhaden Oil	5.20	3.25	0.00
Olive Oil	0.00	2.36	6.20
Wheat Gluten	5.66	5.66	5.66
ARA Oil ¹	0.34	0.28	0.18
DHA Oil ²	3.90	3.50	3.00
Spirulina ³	15.95	16.00	16.00
Naturose ⁴	1.00	1.00	1.00
DICAL	0.85	0.85	0.85
VIT E ⁵	0.08	0.08	0.08
VIT C ⁶	0.09	0.09	0.09
VIT Premix ⁷	0.50	0.50	0.50
Mineral Premix ⁸	0.11	0.11	0.11
Choline Chloride	0.09	0.09	0.09
Lecithin	1.23	1.23	1.24
Ethoxyquin	0.01	0.01	0.01
Total	100.00	100.00	100.00

¹ Vevodar™ by DSM Food Specialties Inc., Parsippany, New Jersey

² DHAsco™ by Martek Biosciences Corp., Columbia, Maryland

³ Spirulina Pacifica® by Cyanotech Corp., Kona, Hawaii

⁴ Naturose® (1.5% Astaxanthin) by Cyanotech Corp., Kona, Hawaii

⁵ Vit E (DL-Acetate liquid form) added directly to oil mixture

⁶ Stay-C® (35% Vit.C) by Hoffman La-Roche, Nutley, New Jersey

⁷ Vitamin pre-mix (LV99.1) manufactured by Roche for OI included: Vitamin A (6000 IU Kg⁻¹), Vitamin D (2000 IU Kg⁻¹), Vitamin E (250 mg Kg⁻¹), Thiamin (40mg Kg⁻¹), Riboflavin (60mg Kg⁻¹), Pyridoxine (60mg Kg⁻¹), D-biotin (0.6mg Kg⁻¹), Niacin (80mg Kg⁻¹), Ca-Panthothenic Acid (180mg Kg⁻¹), Vitamin B₁₂ (0.096mg Kg⁻¹), Folic Acid (6mg Kg⁻¹), Astaxanthin (60mg Kg⁻¹), Menadione (40mg Kg⁻¹), Inositol (400mg Kg⁻¹)

⁸ Mineral mix contained CuSO₄*5H₂O (5.0mg Kg⁻¹), FeCl₃*6H₂O (72mg Kg⁻¹), MnSO₄*H₂O (24mg Kg⁻¹), KI (3.6mg Kg⁻¹), Se (0.25mg Kg⁻¹), and ZnSO₄*7H₂O (100mg Kg⁻¹). CoCl₂*6H₂O (1.2mg Kg⁻¹), and MoNa₂O₄*2H₂O (0.2mg Kg⁻¹)

Table 6.2. Calculated proximate composition and n-3 HUFA levels¹ of treatment diets from Experiment 1.

	Treatment Diet		
	High n-3	Med n-3	Low n-3
% Protein	60.16	60.86	60.19
% Lipid	15.80	15.85	16.03
ARA (mg/gDW) ¹	2.14	1.73	1.06
EPA (mg/gDW) ¹	10.72	8.69	5.29
DHA (mg/gDW) ¹	26.78	22.72	16.67
EPA:ARA	5.02	5.01	5.01
DHA:EPA	2.50	2.62	3.15
Σ(n-3) HUFA %DW	3.83	3.16	2.12

¹ARA, EPA and DHA levels are expressed as mg/g of the diet dry weight.

6.3.1.6. Tank Maintenance. The tanks were routinely siphoned to remove any excess feed or debris and the tank walls and bottoms were scrubbed with a soft pad on a long pole once/week. Additionally, the PVC scaffolding, which served as an artificial “reef” for the fish, was removed once per week and cleaned thoroughly. Approximately twice per month, the fish were removed from their tank and moved to a clean tank, purposefully kept empty to serve as a rotation tank. Following being moved to the clean tank, the old tank would then be scrubbed, rinsed with a chlorine solution, rinsed with freshwater, dried, and then refilled to accommodate another pair.

6.3.1.7. Egg Collection and Sampling. Eggs were collected using modified tank-mounted egg collectors as seen in Figure 6.1. The collectors were modified from those previously described (Chapter 5) to further minimize water turbulence within the collectors, as reduction of turbulence was found to reduce stress on the eggs (see Chapter 2). The inflow into the collector was reduced from 2” to ¾” and a PVC “T” coupled to a PVC 45 degree coupling was used to direct the incoming water below the surface of the water in the collector. This eliminated splashing or air bubbles in the

collector, and allowed the eggs to sit relatively undisturbed at the water's surface in the collector, despite a high flow rate (7-8L/min) of water through the collector. The screen was slid down into position at the end of each day to concentrate eggs in the collector. Each morning, all of the collectors were emptied, by opening the drain valve on the bottom, into individual, clean 1L beakers (and filled to approximately 800ml using clean seawater). The screens were then rinsed and left off the collectors throughout the day, so as to not collect waste and debris on the screens.



Figure 6.1 Photograph of modified egg collector with screen slid into place to concentrate eggs. PVC “T” and 45-degree coupling was used to minimize surface splashing and reduce turbulence in collector.

6.3.1.8. Egg Measurements and Hatch Rate Determination

6.3.1.8.1. Egg Measurements. Following two months of feeding on the treatment diets, eggs from each pair were sampled once per month (20-30 eggs/pair). A sample of approximately 20 eggs from each pair was placed onto a multi-well slide (5 eggs per slide) and photographed using an Olympus Q-Color 3™ digital camera paired with an Olympus CX31 microscope. Images were then measured to the nearest $\pm 0.01 \mu\text{m}$ using Q-Capture Pro™ software. Egg diameter was measured along the body axis and oil droplet diameter was also measured. Mean egg diameter and oil droplet diameter was then determined for each pair using the combined mean from four sampling periods. Treatment means were then calculated based on these individual pair means.

6.3.1.8.2. Hatch Rate Determination. If viable eggs were obtained, samples of eggs (40-80 eggs/pair) from each pair were placed into 4 –8 wells (10 eggs per well) of a 12 well micro-titer plate. Eggs were individually counted into the well by pipetting them from the surface of a beaker into the well chambers. The volume of the well was brought up to 5 ml using clean seawater from the system that the eggs came from. The entire tray was then placed into a 26°C room to incubate and hatch. Hatch rates were calculated by counting the hatched and un-hatched embryos in each well under a dissecting scope. Mean percent hatch for each pair was determined by calculating the number of embryos that hatched in the wells divided by the total number of eggs that were placed into the wells. Treatment means were then calculated by averaging the pair means.

6.3.1.8.3. Day 3 Survival Determination. Survival to day 3 (yolk exhaustion) was determined by placing up to 40 viable, buoyant eggs (120 eggs/pair) into 1L beakers (n=3 beakers per pair). Eggs were individually counted into the beakers containing seawater from the systems from which the eggs came. Each beaker was brought up to exactly 1 liter and then placed into a 26.5°C water bath. The vessels were subjected to a 12h L:12h D photoperiod and left static (no aeration or water exchange) for the 72-hour duration, as maintaining flame angelfish larvae in systems with zero turbulence was found to maximize survival to day 3 (see Chapter 5). Approximately 100,000 cells/ml of microalgae (*T-Isochrysis galbana*) was added to each beaker upon stocking to help maintain water quality. On the morning of day 3 post-hatch, the remaining larvae were counted and percent survival determined.

6.3.2. Experiment 2: Effects of High Dietary n-3 HUFA Level on Flame Angelfish Egg Quality

6.3.2.1. Pair Ranking and Assignment to Treatment Groups. Following three months of feeding on a “mixed” diet regime as described in Chapter 5, flame angelfish pairs from the previous trial (Exp. 1) were ranked according to egg quality criteria. For each pair, means of the preceding 3 months’ data for egg viability, fertilization rates and fecundity were calculated. Pairs were then sorted and assigned treatments as previously described (Exp. 1).

6.3.2.2. Broodstock Husbandry

6.3.2.2.1. Feeding. Flame angelfish were stocked into the experimental system and maintained as described previously (Exp. 1). Broodstock pairs were fed one of four treatment diets to apparent satiation four times daily. The treatment diets consisted of a

formulated low n-3 HUFA diet (Low 2), a medium n-3 HUFA diet (Med 2), a high n-3 HUFA diet (High 2), and a Control diet. The Control diet was administered as described in the previous trial. The composition of the three experimental formulated diets can be found in Table 6.3.

6.3.2.2.2. Diet Formulation and Processing. The diets to be used in this trial were formulated to bracket the n-3 HUFA levels from the previous trial (Exp. 1). Therefore, the Med 2 diet was designed to replicate the n-3 HUFA level of the previous trial's High n-3 diet. Additionally, one lower (Low 2) and one higher (High 2) n-3 HUFA level would be tested. In order to attain a higher total n-3 HUFA level, the total lipid level of the diet needed to be increased. Therefore, the formulation of the diets needed to change slightly from the original formulation in Experiment 1. The calculated proximate composition and HUFA levels of the new diets used in Experiment 2 can be found in Table 6.4. The experimental diets were individually manufactured at OI facilities as previously described for Experiment 1.

Table 6.3. Composition of formulated diets fed to flame angelfish broodstock in Experiment 2.

	Treatment Diet		
	High 2	Med 2	Low 2
Ingredient:	(g/100g)	(g/100g)	(g/100g)
Pollock Meal	31.50	31.50	31.50
Squid Meal	31.50	31.50	31.50
Menhaden Oil	8.00	6.00	4.09
Olive Oil		2.51	5.00
Wheat Gluten	5.66	5.66	5.66
ARA Oil ¹	0.40	0.33	0.30
DHA Oil ²	3.99	3.55	3.00
Spirulina ³	15.00	15.00	15.00
Naturose ⁴	1.00	1.00	1.00
DICAL	0.85	0.85	0.85
VIT E ⁵	0.08	0.08	0.08
VIT C ⁶	0.25	0.25	0.25
VIT Premix ⁷	0.50	0.50	0.50
Mineral Premix ⁸	0.11	0.11	0.11
Choline Chloride	0.09	0.09	0.09
Lecithin	1.07	1.07	1.07
Ethoxyquin	0.01	0.01	0.01
Total	100.00	100.00	100.00

¹ Vevodar™ by DSM Food Specialties Inc., Parsippany, New Jersey

² DHAsco™ by Martek Biosciences Corp., Columbia, Maryland

³ Spirulina Pacifica® by Cyanotech Corp., Kona, Hawaii

⁴ Naturose® (1.5% Astaxanthin) by Cyanotech Corp., Kona, Hawaii

⁵ Vit E (DL-Acetate liquid form) added directly to oil mixture

⁶ Stay-C® (35% Vit.C) by Hoffman La-Roche, Nutley, New Jersey

⁷ Vitamin pre-mix (LV99.1) manufactured by Roche for OI included: Vitamin A (6000 IU Kg⁻¹), Vitamin D (2000 IU Kg⁻¹), Vitamin E (250 mg Kg⁻¹), Thiamin (40mg Kg⁻¹), Riboflavin (60mg Kg⁻¹), Pyridoxine (60mg Kg⁻¹), D-biotin (0.6mg Kg⁻¹), Niacin (80mg Kg⁻¹), Ca-Panthothenic Acid (180mg Kg⁻¹), Vitamin B₁₂ (0.096mg Kg⁻¹), Folic Acid (6mg Kg⁻¹), Astaxanthin (60mg Kg⁻¹), Menadione (40mg Kg⁻¹), Inositol (400mg Kg⁻¹)

⁸ Mineral mix contained CuSO₄*5H₂O (5.0mg Kg⁻¹), FeCl₃*6H₂O (72mg Kg⁻¹), MnSO₄*H₂O (24mg Kg⁻¹), KI (3.6mg Kg⁻¹), Se (0.25mg Kg⁻¹), and ZnSO₄*7H₂O (100mg Kg⁻¹). CoCl₂*6H₂O (1.2mg Kg⁻¹), and MoNa₂O₄*2H₂O (0.2mg Kg⁻¹),

Table 6.4. Calculated composition and HUFA levels¹ of treatment diets from Experiment 2.

	Diet Treatment		
	High 2	Med 2	Low 2
% Protein	57.88	58.50	57.88
% Lipid	18.73	18.84	18.94
ARA (mg/gDW) ¹	2.6	2.15	1.87
EPA (mg/gDW) ¹	13.65	11.57	9.58
DHA (mg/gDW) ¹	30.36	26.09	21.48
EPA:ARA	5.25	5.38	5.11
DHA:EPA	2.22	2.25	2.24
$\Sigma(n-3)$ HUFA %DW	4.6	3.91	3.2

¹ARA, EPA and DHA levels are expressed as mg/g of the diet dry weight.

6.3.2.3. Fatty Acid Analysis of Diets. Samples of each of the diets used in Experiments 1 and 2 were sent to Minnesota Valley Testing Laboratory (MVTl, New Ulm, MN) for fatty acid analyses. Lipid for crude lipid determination was extracted from each of the diet samples by acid hydrolysis according to the Association of Analytical Chemists Official Method (AOAC) 954.02 (AOAC, 2000). After the lipid was extracted, the production of fatty acid methyl esters (FAMES) and subsequent identification and quantification followed AOAC method 996.06 (AOAC, 2000). Results of the analyses were reported as percent of total lipid.

6.3.3. Statistical Analysis

All fecundity, fertility and egg viability data were analyzed using a randomized block design with SYSTAT™ (ver. 11.0). Normality of the data (Shapiro and Wilk, 1965) and homogeneity of the variance (Snedecor and Cochran, 1993) were tested to ensure the assumptions of ANOVA were satisfied. Percent data were transformed

(arcsine) before conducting analysis of variance. Tukey's HSD test (Snedecor and Cochran, 1993) was used to determine significant differences ($p < 0.05$) between the treatment means.

6.4. Results

6.4.1. Experiment 1

6.4.1.1. Fatty Acid Analysis. The fatty acid composition of the Control diet and three experimental diets are given in Table 6.5 and Appendix Table A.3. The resulting experimental diets had three levels of n-3 HUFA (Table 6.6) ranging from 1.84 to 3.63%. In an effort to maintain a constant DHA:EPA:ARA ratio, as recommended by Sargent et al. (1999), ARA level increased with increasing n-3 HUFA level from 1.13mg/g to 2.32mg/g (DW). However, targeted DHA:EPA:ARA ratios of 10:5:1 were not conserved across treatment diets and were only approximated in the Med n-3 and High n-3 diets.

Table 6.5. Fatty acid composition of treatment diets in Experiment 1. Values are expressed as percent (%) of total lipid.

Fatty Acid Profiles (%)

	Control Diet¹	High n-3 Diet	Med n-3 Diet	Low n-3 Diet
14:0	3.56	8.67	6.30	3.37
16:0	24.35	21.51	20.21	18.62
16:1	3.19	6.95	4.86	2.00
18:0	5.86	4.17	4.03	3.93
18:1n-9	15.32	17.34	28.59	44.24
18:2n-6	11.44	7.62	8.48	9.69
18:3n-3	3.76	1.19	1.07	0.91
20:1	1.86	1.26	1.03	0.84
20:4n-6 (ARA)	1.33	1.49	1.22	0.70
20:5n-3 (EPA)	11.32	7.14	5.42	2.67
22:5n-3	0.81	1.05	0.75	0.26
22:6n-3 (DHA)	13.21	15.16	12.64	8.76
Others	2.19	4.53	3.85	2.92
Σ n-3	29.34	24.74	19.98	12.67
Σ n-6	13.52	9.63	10.07	10.55
n-3:n-6	2.17	2.57	1.98	1.20
Σ n-3 HUFA	25.58	23.55	18.91	11.76
DHA:EPA	1.17	2.12	2.33	3.28
EPA:ARA	8.51	4.78	4.45	3.83
DHA:EPA:ARA	9.9:8.5:1	10.2:4.8:1	10.4:4.4:1	12.5:3.8:1

¹ Based on compilation of Raw diet and Spectrum diet analyses. Calculated by addition of 65% of Raw diet values and 35% of Spectrum diet values as offered daily (dry weight basis).

Table 6.6. Composition and HUFA levels¹ of Control diet and formulated treatment diets from Experiment 1.

	Control	High n-3	Med n-3	Low n-3
% Protein	58.34 ²	62.49	62.15	62.04
% Lipid	10.55 ²	15.94	15.90	15.96
ARA (mg/gDW)	1.40	2.37	1.90	1.13
EPA (mg/gDW)	11.94	11.39	8.61	4.26
DHA (mg/gDW)	13.94	24.19	20.09	13.98
Σ (n-3) HUFA % of DW ³	2.69	3.63	2.97	1.84

¹ ARA, EPA and DHA levels are expressed as mg/g of the diet dry weight and were calculated from [respective values (Table 6.5) x 10] X [lipid level (Table 6.6)].

² Based on compilation of Raw diet and Spectrum diet analyses. Calculated by addition of 65% of Raw diet values and 35% of Spectrum diet values as offered daily (dry weight basis).

³ Calculated from [Σ n-3 HUFA (Table 6.5)] X [lipid level (Table 6.6)]

6.4.1.2. Effects of Diet on Egg Quality

6.4.1.2.1. Effects of Diet on Egg Biochemistry.

Egg samples were taken throughout the course of this study and have been sent to the Center of Marine Biotechnology (COMB), University of Maryland Biotechnology, Baltimore, Maryland for fatty acid analysis (as described in Chapter 5). However, those samples were not processed in time for inclusion in this dissertation. When processed, those data will be included in publications that result from these experiments.

6.4.1.2.2. Effects on Fecundity.

The effects of treatment diet on total egg output are presented in Figure 6.2. Within the first month of feeding, fecundity was significantly lower from pairs that received the Med n-3 diet than pairs that received the High n-3 diet. This trend continued for the duration of the experiment. Pairs that received the Low n-3 diet exhibited decreased fecundity from month 2 onward and produced significantly fewer eggs than pairs that received the High n-3 and Control diets from month 3 onward.

Fecundity was not significantly different between pairs that received the Control diet and the High n-3 diet for the duration of the experiment. After four months of feeding on the treatment diets, pairs that received the Med and Low n-3 diets fecundity leveled off at approximately 750 eggs per spawn. This was significantly lower than pairs that received either the Control diet or the High n-3 diet, which were producing 1200 to 1500 eggs per spawn, respectively.

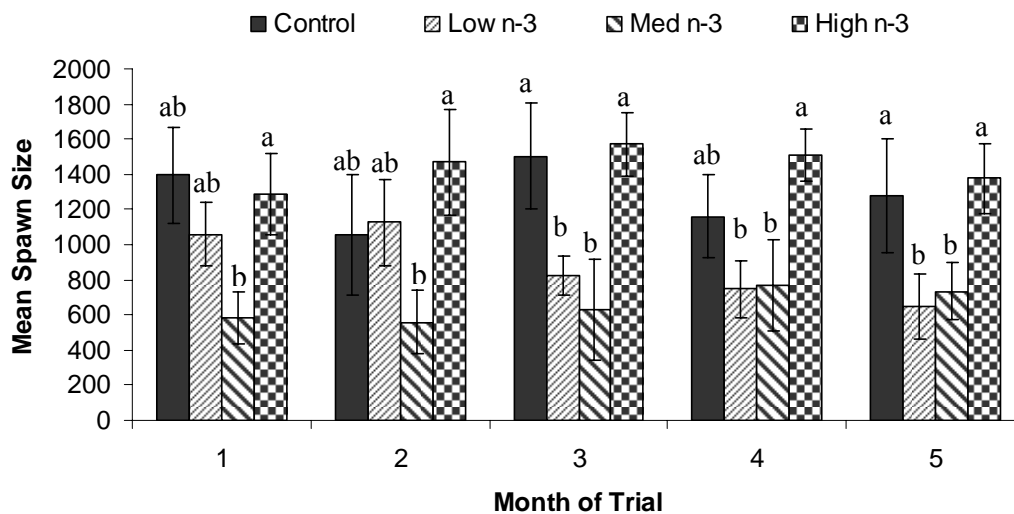


Figure 6.2. Flame angelfish mean daily egg production during Experiment 1. Values are monthly means ($n=4$) \pm standard error. Means with the same superscript are not significantly different ($p>0.05$).

6.4.1.2.3. Effects on Egg Fertilization. The effects of treatment diet on egg fertilization rates are presented in Figure 6.3. As with fecundity, fertilization rates were lower within the first month of feeding among pairs that received the Low and Med n-3 diets compared to pairs that received the Control or High n-3 diets, however these differences were not significant ($p>0.05$) until the third month of the experiment. Fertilization rates among pairs that received the Low and Med n-3 diets dropped from an

average of 50% prior to the start of the trial to less than 10% by the end of the third month. Contrary to those treatments, pairs that received the Control and High n-3 diets maintained significantly higher fertilization rates of 50% and 45%, respectively, throughout the first two months. Fertilization rates for all treatment groups fell significantly over the course of the trial, particularly during month 3 of the experiment, when only the High n-3 treatment produced significant numbers of fertile eggs. For the last two months of the experiment, pairs that received the Control and High n-3 diets maintained significantly higher fertilization rates than pairs that received either the Low or Med n-3 treatment diets.

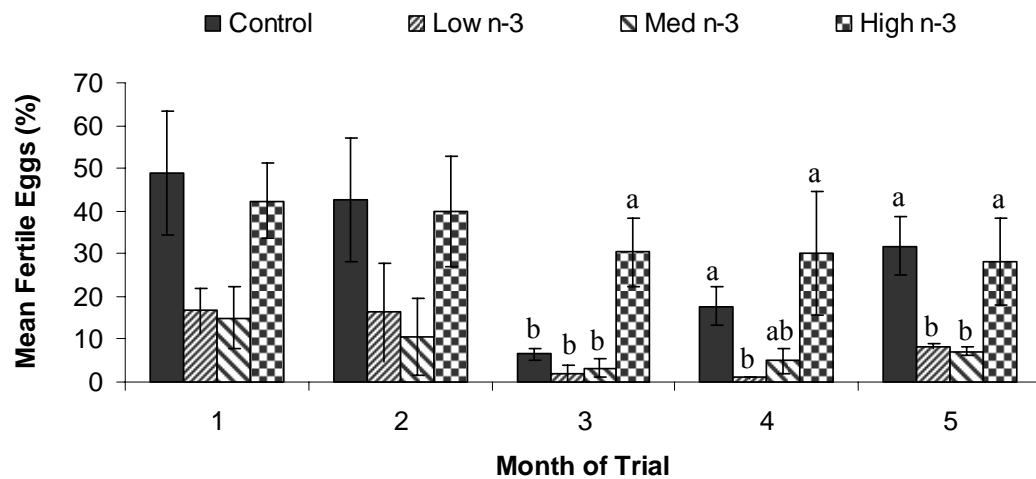


Figure 6.3. Flame angelfish mean daily egg fertilization rates during Experiment 1. Values are monthly means ($n=4$) \pm standard error. Means with the same superscript are not significantly different ($p>0.05$).

6.4.1.2.4. Effects on Egg Viability. The effects of treatment diet on egg viability are presented Figure 6.4. As with fecundity and fertilization rates, viability rates were lower within the first month of feeding among pairs that received the Low and Med n-3 diets compared to pairs that received the Control or High n-3 diets, although these differences were not significant ($p>0.05$) until month 3. Viability rates for pairs that received the Low and Med n-3 diets dropped from an average of 25% prior to the start of the trial to less than 10% by the end of the first month. Contrary to those treatments, pairs that received the Control and High n-3 diets maintained higher viability rates of approximately 22% throughout the first two months. As with fertilization rates, viability rates for all treatment groups fell significantly over the course of the trial, although the pairs that received the Control and High n-3 treatments maintained significantly higher viability rates than pairs that received the Low or Med n-3 diets.

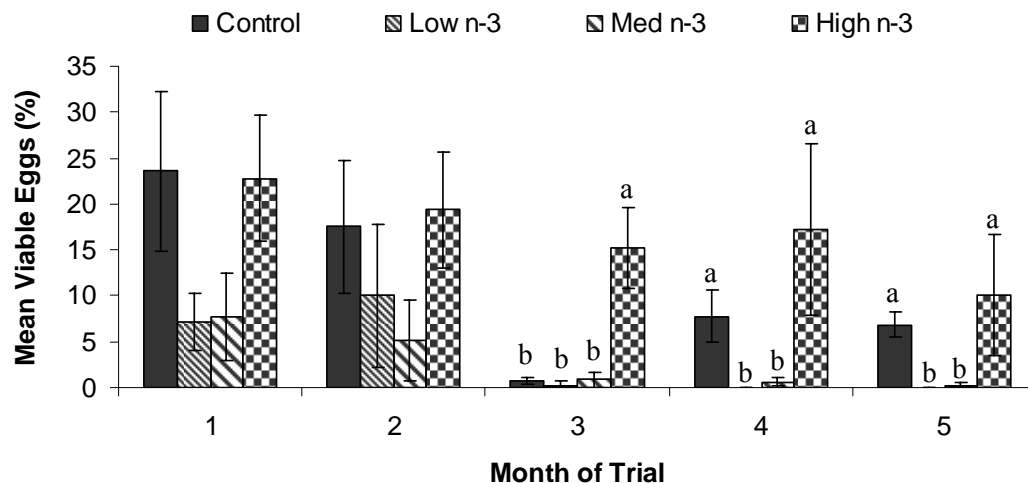


Figure 6.4. Flame angelfish mean daily egg viability rates during Experiment 1. Values are monthly means ($n=4$) \pm standard error. Means with the same superscript are not significantly different ($p>0.05$).

6.4.1.2.5. Effects on Egg Size, Percent Hatch, Larval Size and Percent

Survival. The effects of diet type on egg and larval size and survival characteristics are presented in Table 6.7. Egg diameter and oil globule diameter were not significantly affected by diet type; however percent hatch was significantly affected by diet type, as High n-3 diet treated pairs exhibited the highest hatch rates (65.04%). Pairs that received the Low n-3 and Med n-3 diets failed to produce any viable eggs, and thus eggs did not hatch. Pairs that received the Control diet exhibited mean hatch rates of 50.60%. Mean length at hatch and on day 3 post-hatch was not significantly different among the pairs that received either the control or High n-3 diets. Mean survival to day 3 post-hatch was also not significantly different among these treatments and averaged 73% for each treatment group.

Table 6.7. Flame angelfish mean egg and larval size characteristics, percent hatch and percent survival of larvae to day 3 post-hatch from broodstock fed different diets in Experiment 1.

Treatment Diet	Control	High n-3	Med n-3	Low n-3
Egg Diameter (μm)	691.48 \pm 9.54	676.60 \pm 10.25	696.27 \pm 13.73	704.70 \pm 13.93
Oil Globule Diameter (μm)	154.97 \pm 2.69	156.39 \pm 5.37	154.33 \pm 5.55	160.42 \pm 6.80
Hatch (%)	50.6 \pm 5.85 ^a	65.04 \pm 6.45 ^b	0.00 ^c	0.00 ^c
Length at Hatch (μm)	1560.82 \pm 65.85	1582.11 \pm 32.72	n/a	n/a
Length at day 3 (μm)	2281.80 \pm 140.80	2368.00 \pm 65.05	n/a	n/a
Survival to day 3 (%)	73 \pm 10.7	73 \pm 17.6	n/a	n/a

Values are reported as means \pm standard error.

Different superscripts within the same row indicate significant difference ($p < 0.05$).

6.4.2. Experiment 2

6.4.2.1. Fatty Acid Composition of Diets. The fatty acid composition of the Control diet and the three experimental diets are given in Table 6.8 and Appendix Table A.4. The resulting experimental diets had three levels of n-3 HUFA (Table 6.9) ranging from 2.68 to 4.34%. In an effort to maintain a constant DHA:EPA:ARA ratio, as recommended by Sargent et al. (1999), ARA level increased with increasing n-3 HUFA level and ranged from 2.41mg/g to 2.91mg/g (DW). Targeted DHA:EPA ratios of approximately 2:1 were conserved across treatment diets. However, targeted EPA:ARA ratios of 5:1 were not achieved and instead ranged from 3.2 to 4.37.

Table 6.8. Fatty acid composition of treatment diets in Experiment 2.
Values are expressed as % of total lipid.

Fatty Acid Profiles (%)

	Control Diet¹	High 2 Diet	Med 2 Diet	Low 2 Diet
14:0	3.56	7.77	6.13	4.68
16:0	24.35	20.97	19.48	18.21
16:1	3.19	6.94	5.46	4.17
18:0	5.86	4.27	4.12	4.05
18:1n-9	15.32	19.54	29.26	38.19
18:2n-6	11.44	6.64	7.52	8.11
18:3n-3	3.76	1.35	1.22	1.07
20:1	1.86	1.38	1.14	1.03
20:4n-6 (ARA)	1.33	1.64	1.44	1.38
20:5n-3 (EPA)	11.32	7.19	5.75	4.41
22:5n-3	0.81	1.31	0.99	0.72
22:6n-3 (DHA)	13.21	15.74	12.98	10.12
Others	2.19	3.43	3.08	2.65
 Σn-3	 29.34	 25.83	 21.12	 16.45
Σn-6	13.52	8.76	9.39	9.82
n-3:n-6	2.17	2.95	2.25	1.68
 Σn-3 HUFA	 25.58	 24.48	 19.90	 15.38
 DHA:EPA	 1.17	 2.19	 2.26	 2.29
EPA:ARA	8.51	4.37	3.99	3.20

¹ Based on compilation of Raw diet and Spectrum diet analyses. Calculated by addition of 65% of Raw diet values and 35% of Spectrum diet values as offered daily (dry weight basis).

Table 6.9. Composition and n-3 HUFA levels¹ of Control diet and formulated treatment diets from Experiment 2.

	Control	High 2	Med 2	Low 2
% Protein	58.34 ²	58.01	57.89	59.18
% Lipid	10.55 ²	17.75	17.51	17.47
ARA (mg/gDW)	1.40	2.91	2.52	2.41
EPA (mg/gDW)	11.94	12.76	10.06	7.70
DHA (mg/gDW)	13.94	27.93	22.72	17.67
Σ (n-3) HUFA %DW ³	2.69	4.34	3.48	2.68

¹ ARA, EPA and DHA levels are expressed as mg/g of the diet dry weight and were calculated from [respective values (Table 6.8) \times 10] \times [lipid level (Table 6.9)]

² Based on compilation of Raw diet and Spectrum diet analyses. Calculated by addition of 65% of Raw diet values and 35% of Spectrum diet values as offered daily (dry weight basis).

³ Calculated from [Σ n-3 HUFA (Table 6.8)] \times [lipid level (Table 6.9)]

6.4.2.2. Effects on Fecundity. The effects of diet on flame angelfish fecundity are presented in Figure 6.5. For the majority of the experiment, there were no significant differences detected between treatment groups. From week 3 to week 4, fish in the Control treatment produced significantly more eggs (approximately 1600, and 1200 eggs per spawn respectively) than fish from any of the other treatments (producing approximately 800 eggs per spawn). However, in week 5, fecundity among Control treatment pairs decreased and was not significantly different from the other treatments. From week 6 onward, fecundity increased in all treatment groups as all of the pairs were returned to the Control diet regimen. From that point on, no significant differences were detected between treatments.

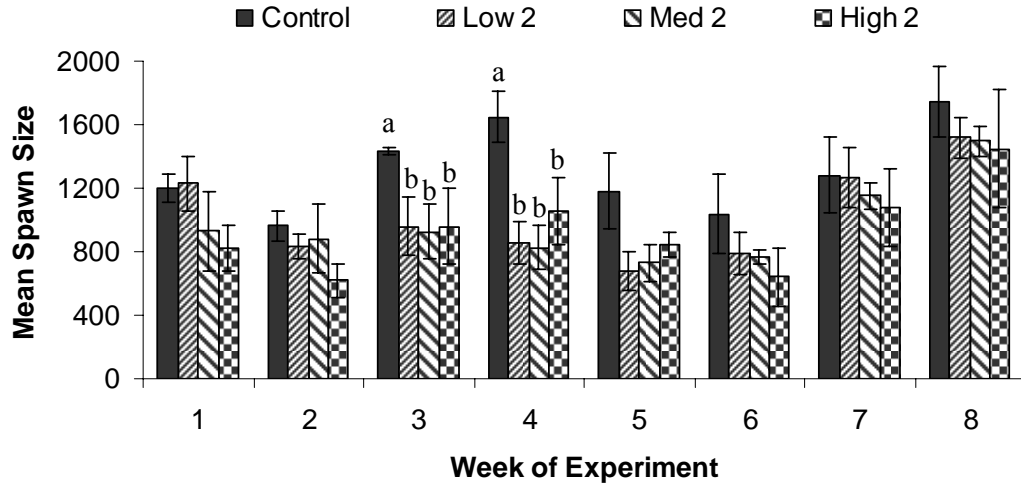


Figure 6.5. Flame angelfish weekly mean fecundity from Experiment 2. Values are weekly means ($n=4$) \pm standard error. At week 6, all pairs were returned to Control diet regime for duration of study. Means with the same superscript are not significantly different ($p>0.05$).

6.4.2.3. Effects on Fertilization. The effects of diet type on flame angelfish egg fertilization rate are presented in Figure 6.6. During weeks 1 and 2, mean fertilization rates dropped in pairs that received the experimental diets, where as pairs that received the Control diet maintained relatively constant fertilization rates for the duration of the first 5 weeks (approximately 40%). Initially, this decline appeared to be dose-dependent, with the High 2 diet exhibiting the most rapid decline. However, the majority of observed differences in fertility over the first 3 weeks were not statistically significant ($p>0.05$). At the end of the 5th week of the experiment, pairs that received the Low 2 and Med 2 diets exhibited mean fertilization rates of 16% and 11% respectively. Pairs that received the High 2 diet exhibited mean fertilization rates of 28%. Pairs that received the Control diet exhibited mean fertilization rates of 35%. From week 6 onward, all pairs were returned to

the Control feeding regime and all pairs were not significantly different with regard to mean fertilization rates from that point forward.

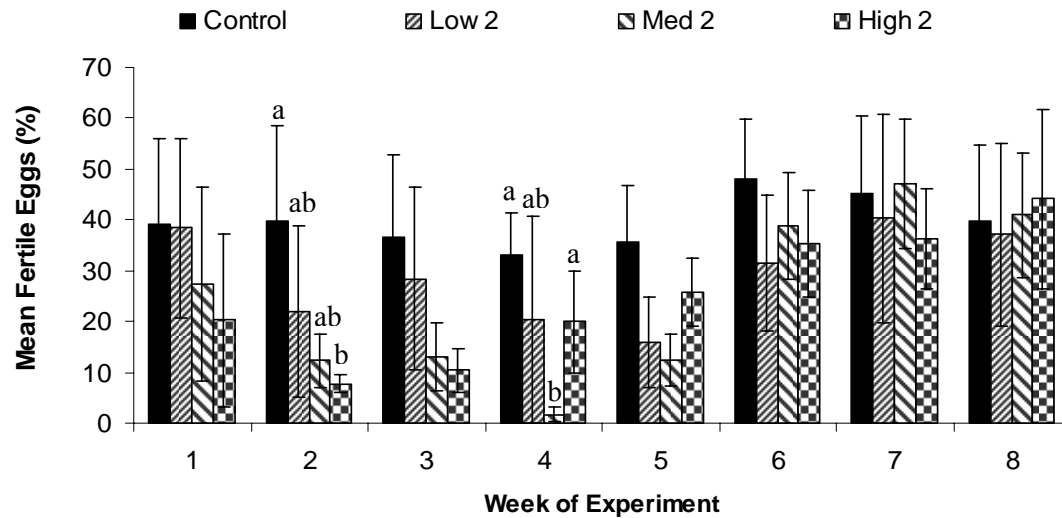


Figure 6.6. Flame angelfish weekly mean egg fertilization rate from Experiment 2. Values are weekly means ($n=4$) \pm standard error. At week 6, all pairs were returned to Control diet regime for duration of study. Means with the same superscript are not significantly different ($p>0.05$).

6.4.2.4. Effects on Egg Viability. The effects of diet type on flame angelfish egg viability are shown in Figure 6.7. During weeks 1 and 2, mean egg viability dropped in all pairs that received the experimental diets, where as pairs that received the Control diet maintained relatively constant egg viability for the duration of the first 5 weeks (approximately 20-30%). During week 2, Control treatment pairs exhibited significantly higher egg viability than any of the pairs receiving the experimental diets. At the end of the 5th week of the trial, pairs that received the Low 2 and Med 2 diets exhibited significantly lower mean egg viability of 1% and 0% respectively than pairs that received the High 2 diet (10%) and pairs that received the Control diet (20%). From week 6

onward, all pairs were returned to the Control feeding regime and all pairs were not significantly different with regard to egg viability from that point forward.

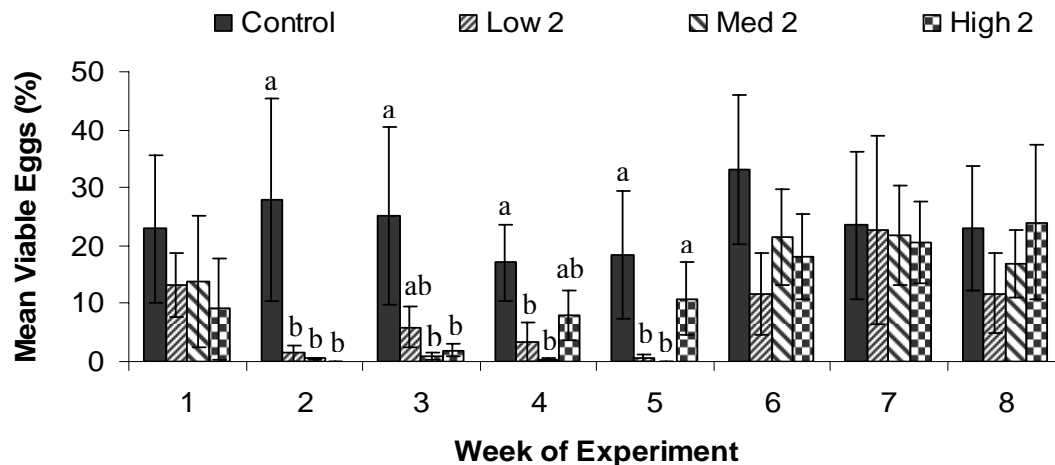


Figure 6.7. Flame angelfish weekly mean egg viability rates from Experiment 2. Values are weekly means ($n=4$) \pm standard error. At week 6, all pairs were returned to Control diet regime for duration of study. Means with the same superscript are not significantly different ($p>0.05$).

6.5. Discussion

The decreased fertilization and egg viability rates observed during Experiment 1 can likely be attributed to the implemented change in chlorination protocol for the use of ocean water, which increased both the level of chlorination and exposure duration (see Chapter 4). Experiment 1 was initiated in April 2006 (month 1) and ran until August 2006 (month 5). Therefore, the experimental period overlapped the occurrence of this new chlorination protocol. It is likely that the observed negative affects on fertilization and egg viability observed in the early stages of those experiments (Chapter 4) were mirrored in current experiment. However, since only a small portion of the system water was exchanged (10% weekly) during this diet experiment, we did not observe the same

rapid effects on egg quality that were observed in the experiments of Chapter 4. Instead, we observed a more gradual, system-wide affect on egg quality, resulting in an overall-reduction of fertilization (Figure 6.8) and egg viability (Figure 6.9).

Prior to the switch in ocean water sterilization protocol (Fig. 6.8 dashed lines), mean monthly egg fertilization rates were near 80% for pairs on the Control treatment diet and approximately 60% for all pairs combined. Data from pairs that had been receiving the Control diet (control mean) throughout the observation period are provided to illustrate that the reduction of fertile eggs (Fig. 6.8) and viable eggs (Fig. 6.9) occurred independently of the diet experiments, as their diet did not change.

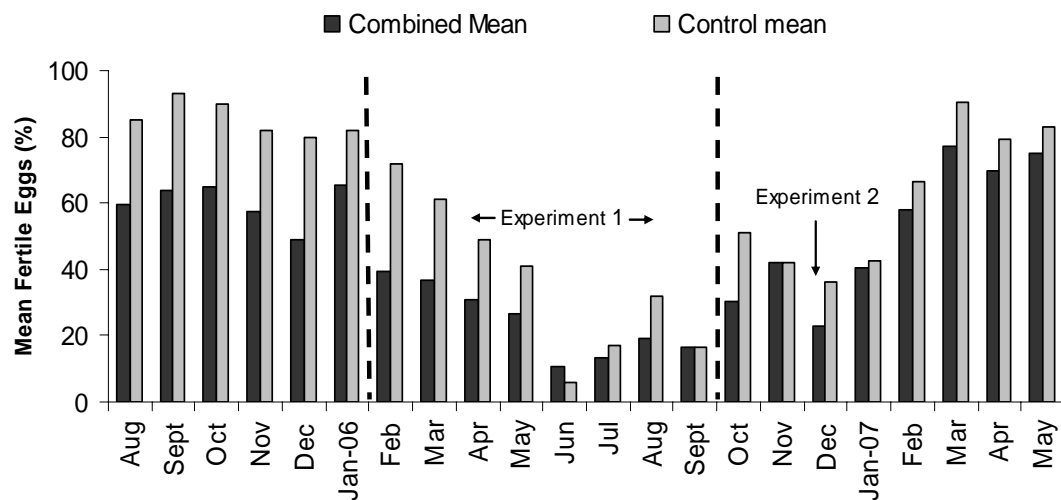


Figure 6.8. Flame angelfish mean egg fertilization rate of control treatment pairs (n=4 pairs) and combined (all treatments including control) mean (n=18 pairs) from August 2005 to May 2007. Dashed lines indicate the duration of altered chlorination protocols. Change in chlorination protocol was initiated in Feb. 2006 lasting through Sep. 2006. In late Sep, 2006, 100% of the system water was exchanged with water chlorinated at the original 2005 level.

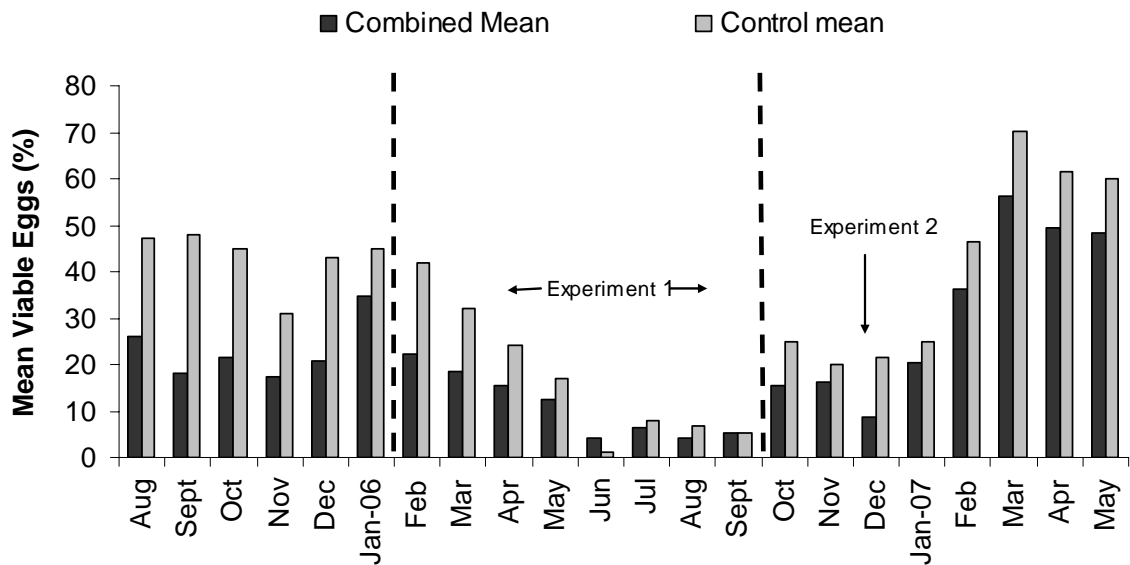


Figure 6.9. Flame angelfish mean egg viability rate of control treatment pairs (n=4) and combined treatments mean (n=18) from August 2005 to May 2007. Dashed lines indicate the duration altered chlorination protocols. Change in chlorination protocol was initiated in Feb. 2006 lasting through Sep. 2006. In late Sep. 2006, 100% of the system water was exchanged with water chlorinated at the original 2005 level.

Mean monthly egg viability rates (Fig. 6.9) were near 50% for pairs on the Control treatment and were increasing to approximately 40% for all pairs combined prior to the switch in ocean water sterilization protocol. As observed in the fertilization rates, the gradual addition of new water, sterilized with a higher level of chlorine, also reduced egg viability rates to less than 10% in all pairs by June 2006. Furthermore, in June 2006, we experienced temperature control complications brought about by the failure of the OMO system's chiller unit. This failure allowed the system's water temperature to fluctuate between 26-29°C daily. Normally, the system's water temperature is maintained tightly at 26-27°C. These larger than normal, daily temperature fluctuations also had a negative impact on the fish' spawning behaviors, as many pairs temporarily ceased

spawning during that time. Temperature control was restored to the system within one week of malfunction. However, its negative impacts on spawning were observed for many weeks following.

Once it was suspected that the chlorination protocol was affecting egg quality in the other experiments (Chapter 4), all the OMO system water was exchanged with new water, treated with the original chlorination protocol. However, this exchange followed the conclusion of Experiment 1, and took place during the pairs' "re-conditioning" period. This exchange of new water brought about a rapid increase in egg fertilization rate (Fig 6.8), which is continuing to date. Additionally, egg viability has also rapidly recovered (Fig.6.9) with mean egg viability approaching 40-50% of eggs produced.

Despite the challenges encountered, within the first month of Experiment 1, effects of diet type could be observed on flame angelfish fecundity, fertilization rate and egg viability. Pairs that received the Med n-3 diet exhibited dramatically reduced fecundity within the first month of the trial; whereas the negative effects on fecundity of the pairs that received the Low n-3 diet were more gradual. However, from month 3 onward, pairs that received the High n-3 and Control diets produced significantly more eggs than pairs fed either the Low n-3 or Med n-3 diets.

The effects of diet type on fertilization rate were more consistent among pairs that received the Low n-3 and Med n-3 diets, where within the first month mean monthly fertilization rates dropped in those treatments to less than 20%. Mean monthly egg viability responded equally fast, dropping to <10% in the first month in the Low n-3 and Med n-3 treatment groups. Throughout the trial, pairs fed the High n-3 and Control diets exhibited significantly higher egg fertilization and egg viability rates than pairs that were

fed the other treatment diets. Furthermore, pairs that received the High n-3 diet did not differ significantly, with regard to fecundity, fertilization or egg viability, from pairs fed the Control diet. However, both fertilization rate and egg viability decreased significantly within these treatment groups over the course of the experiment.

Diet type did not affect egg diameter or oil globule size among treatments, but did significantly affect hatch rate. Hatch rates of approximately 50% were obtained from pairs on the Control diet. However, this level is much lower than we have recorded in the past, as normal hatch rates are expected to average 80% (Chapter 5). Pairs of fish fed the High n-3 diet exhibited hatch rates that averaged 65%. This level, though still lower than expected, was significantly greater than from pairs that received the Control diet, over the same time period. Larval size at hatch, larval size at day 3 post-hatch (yolk-exhaustion) and survival to day 3 post-hatch was not significantly different between fish fed the Control and High n-3 diets. The pairs that were fed the Low n-3 and Med n-3 diets did not produce viable eggs. Therefore eggs from both of those treatments did not hatch, and no larval size measurements or survival estimations were calculated.

During month 3 (June, 2006) of Experiment 1, pairs that received the Control diet exhibited an uncharacteristic, sharp decline in fertilization rate and egg viability. It is probable that the coupled stressors of temperature fluctuation and elevated chlorination treatment of the system water may have caused this dramatic reduction in egg quality among those pairs. However, pairs that received the High n-3 diet continued to exhibit relatively good fertilization rates and produced significantly greater numbers of viable eggs during that same period. Therefore, that time period served as an unintentional “stress-test” in which fish that received the highest level of HUFA (dry weight basis)

performed best. These results are similar to other reported observations (reviewed in Bell and Sargent, 2003; Furuita et al., 2003) where increased HUFA level correlated with improved stress resistance. Although investigation of improved stress resistance as a function of diet was not intended, this species would be amenable to further study in this area, as spawning data and egg quality information can be collected and assessed quickly.

As only the High n-3 diet (Experiment 1) performed similar to the Control diet, the objective of Experiment 2 was to determine if higher dietary n-3 HUFA level would positively or negatively affect flame angelfish egg quality. We anticipated that fish that received the Med 2 diet would have performed comparably to fish that had received the High n-3 diet in Experiment 1, since they were formulated to contain approximately the same n-3 HUFA level, and contained nearly the same dietary formulation. However, shortly after Experiment 2 began, it became clear that all of the fish were not performing well on any of the new diets. Furthermore, below average feeding response by the fish on the formulated diets indicated that the diets were less palatable than the diets made for Experiment 1. It is possible that the increased lipid content of the diets in Experiment 2 reduced their palatability to the flame angelfish.

Egg fertilization rates (Fig. 6.6) and particularly egg viability (Fig. 6.7) rapidly decreased in all pairs that received the formulated diets. Furthermore, pairs that received the Control diet were also exhibiting lower than expected, and highly variable, fertilization and egg viability rates. It is possible that the reduced spawning performance observed among those pairs was caused, in part, by delayed re-conditioning following the change of chlorination protocols. Mean fertilization rates (Fig. 6.8) and egg viability rates (Fig. 6.9) of Control treatment pairs did not return to expected values until February 2007

indicating that the experimental period (Experiment 2) preceded these fish fully recovering. Observed poor feeding response of the fish, coupled with reduced spawning performance on all the formulated diets, caused us to end the experiment. However, once the pairs were placed back onto the Control diet (week 6) we observed rapid recovery of spawning characteristics.

In summary, although the results of Experiment 1 were confounded by the affects of temperature and the altered chlorination protocol, some important conclusions can be determined from the results. First, flame angelfish readily consumed and spawned on all the test diets presented to them. Diets formulated for the current experiments resulted in significantly greater reproductive performance than in previous diets tested (Chapter 5). Second, results from Experiment 1 indicated that pairs receiving the High n-3 diet spawned as well, or in some cases, better than pairs receiving the Control diet, indicating that n-3 HUFA level is important in flame angelfish reproduction. This was particularly apparent during month 3 of the trial, when the only viable eggs obtained were from pairs on the High n-3 diet. This result is particularly exciting, as the creation of a formulated “control” diet will greatly accelerate our ability to test individual dietary factors. Furthermore, using the formulation and n-3 HUFA level of the High n-3 diet, we now have a reference point by which to guide future diet development.

Although Experiment 2 was ended early due to poor feed consumption and spawning characteristics, it was determined that flame angelfish spawning performance could react quickly to dietary changes, as originally anticipated. Within only two of weeks of diet transition, differences in egg production, fertilization and viability could be detected. This response is different from what was observed following the diet

experiment in Chapter 5, where the fish took 4 months to “recover” from diet affects. It is possible that the reason for the long recovery period following that experiment was due to inadequate nutrient composition of those experimental diets, and the fact that the fish were maintained on those diets for a prolonged period (8 months). The fish in Experiment 2 demonstrated the ability to rapidly adapt back to “normal” spawning performance given suitable environmental parameters and diet. Therefore, future diet trials with this species may only need to be planned for 6-8 weeks, rather than 6 months. This shorter trial duration, if possible, would allow for much more rapid assessment of important dietary components and how they interact with angelfish reproduction. Future experiments could utilize the High n-3 diet formulation to investigate the roles of the individual HUFA in order to better understand their roles in marine fish egg production and egg quality.

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Chapter 7

SIGNIFICANCE AND FUTURE WORK

In this dissertation I have identified the flame angelfish (*Centropyge loriculus*) as an ideal model for testing the effects of environmental and nutritional factors on egg and larval quality in marine fish. This species is readily available (via the aquarium industry), acclimates well to captivity, conditions for spawning quickly and is capable of sustained daily egg production over many years. Furthermore, results of this project have confirmed that this species is highly amenable to broodstock nutrition research due to its unique spawning strategy and rapid exhibition of dietary effects on egg quality characteristics. This research also led to the development of a formulated diet that was capable of supporting long-term health and excellent egg quality in this species. The reference diet developed herein can now be utilized to begin testing the effects of individual dietary components on factors such as egg production and egg quality. Therefore, the continuation of this line of research will not only benefit those interested in marine ornamental species, but also those studying marine fish nutrition in general.

In addition to demonstrating the suitability of this species to fish nutrition research, this project established a comprehensive dataset of baseline spawning performance for flame angelfish. During this evaluation, significant insights were gained as to the factors that affect egg production and egg quality in this species. For nearly two years, a broodstock population of 18 pairs continually produced approximately 30,000 eggs daily, with combined monthly totals approaching 1 million eggs per month. For the first time, commercial-scale production of eggs from a pelagic-spawning marine

ornamental species was achieved, indicating that this species is capable of providing sufficient eggs for production-scale larviculture research efforts. Thus one of the critical bottlenecks (limitation of eggs and larvae) to the successful culture of marine ornamental species has been overcome.

It is anticipated that the continued development of commercial-scale egg production will facilitate the larviculture methods necessary to commercialize the culture of this species, and inevitably lead to the development of culture methods for other marine ornamental species in the future. Therefore, flame angelfish could also serve as invaluable models for the development of larviculture technologies that could then be applied to numerous other species, which exhibit similar egg and larval characteristics. Future research utilizing flame angelfish should focus on the critical early life stages and the development of hatchery technologies for successfully rearing the small, fragile larvae of this species. As the vast majority of marine ornamental species exhibit similar egg and larval characteristics, it is reasonable to presume that even small steps forward in the culture of this species will translate into major milestones for the marine ornamental aquaculture industry in the years to come.

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Appendix

FATTY ACID PROFILES OF FLAME ANGELFISH DIETS AND EGGS

Table A.1. Complete fatty acid profiles of flame angelfish broodstock diets tested in Chapter 5. Values are expressed as % of total fat.

Saturates	Raw Diet	Herbivore Diet	Carnivore Diet	Spectrum Diet
C4:0	0.41			
C6:0	0.03			
C8:0	0.02			
C10:0	0.06			
C12:0	0.10	0.05	0.10	0.25
C13:0				0.06
C14:0	1.97	3.64	6.15	6.50
C15:0	0.33	0.35	0.71	0.47
C16:0	24.75	20.94	25.38	23.61
C17:0	0.55		0.69	0.39
C18:0	6.64	4.69	5.56	4.41
C20:0	0.24	0.28	0.35	0.30
C21:0	0.05		0.07	0.05
C22:0	0.24	0.34	0.32	0.25
C23:0	0.32			0.17
C24:0	0.20	0.25	0.16	
Total Saturates	35.9	30.54	39.48	36.45

Monounsaturates	Raw Diet	Herbivore Diet	Carnivore Diet	Spectrum Diet
C14:1	0.04		0.03	0.07
C15:1				
C16:1	1.91	4.71	7.76	5.57
C17:1		0.60		
C18:1n-9	12.87	16.14	18.19	19.88
C20:1	2.20	0.94	4.53	1.24
C22:1	0.12	0.18	0.69	0.36
C24:1	0.21	0.21	0.78	
Total Monounsaturates	17.35	22.77	31.98	27.12

Polyunsaturates	Raw Diet	Herbivore Diet	Carnivore Diet	Spectrum Diet
C18:2n-6	9.43	30.68	11.82	15.16
C18:3n-4	0.04	0.46	0.12	0.25
C18:3n-3	4.55	3.79	2.15	2.3
C20:2n-6	0.76	0.22	0.36	0.23
C20:3n-6	0.18	0.11	0.12	0.17
C20:3n-3	0.26	0.15	0.17	0.19
C20:4n-6 (ARA)	1.59	0.41	0.58	0.85
C20:5n-3 (EPA)	12.36	4.71	5.54	9.40
C22:2	0.05		0.04	
C22:3	0.06	0.07	0.12	0.05
C22:4	0.05	0.05	0.07	0.18
C22:5	0.77	1.00	1.16	0.89
C22:6n-3 (DHA)	16.68	4.69	6.30	6.75
Total Polyunsaturates	46.75	46.33	28.54	36.43

Table A.2. Complete fatty acid profiles of eggs from flame angelfish broodstock fed different diets in Chapter 5. Values are expressed as % of total fat.

	Raw		Spectrum		OI Carnivore		OI Herbivore	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
C6:0	0.95	0.00	0.32	0.00	1.14	0.00	0.98	0.00
C8:0	-	-	0.15	0.00	-	-	-	-
C10:0	0.24	0.07	0.37	0.00	0.13	0.00	0.28	0.00
C12:0	0.46	0.20	0.35	0.00	0.43	0.00	0.23	0.00
C12:1	-	-	0.62	0.00	-	-	-	-
C14:0	2.23	0.06	2.44	0.57	2.07	0.48	1.60	0.32
C14:1	0.42	0.16	0.48	0.12	0.34	0.04	1.09	0.80
C16:0	21.79	0.79	19.07	0.73	19.32	1.87	15.87	1.14
C16:1	5.86	0.04	6.55	0.39	13.42	2.24	4.29	0.61
C16:2n-4	0.34	0.13	0.64	0.10	0.82	0.06	0.34	0.03
C17:0	0.62	0.06	0.50	0.09	0.62	0.03	0.64	0.13
C16:3n-4	0.46	0.08	0.44	0.12	0.68	0.34	0.49	0.04
C16:4n-1	-	-	0.10	0.00	-	-	-	-
C18:0	6.90	0.27	7.64	1.45	7.53	0.43	7.50	0.44
C18:1ab	10.15	0.14	14.22	3.94	10.78	0.54	13.07	0.80
C18:1c	3.51	0.11	5.79	0.17	6.29	0.54	5.16	0.80
C18:2n-6	5.88	1.03	8.64	0.92	5.30	0.50	19.01	1.05
C18:2n-4	0.15	0.01	0.37	0.03	0.33	0.05	0.29	0.00
C18:3n-4	0.24	0.02	0.44	0.03	0.29	0.04	0.24	0.01
C18:3n-3	0.89	0.13	1.20	0.08	0.99	0.04	1.79	0.13
C18:4n-3	0.32	0.07	1.12	0.66	0.68	0.06	0.40	0.09
C18:4n-1	0.15	0.02	0.34	0.03	0.15	0.00	0.25	0.04
C20:0	0.27	0.01	0.25	0.03	0.22	0.06	0.24	0.03
C20:1ab	0.68	0.09	0.53	0.20	0.65	0.13	0.51	0.03
C20:2n-6	0.71	0.05	0.61	0.05	0.33	0.02	1.20	0.04
C20:3n-6	0.37	0.02	0.57	0.20	0.76	0.12	0.43	0.02
C20:4n-6 (ARA)	2.82	0.41	2.87	0.80	6.67	1.64	1.29	0.25
C20:3n-3	0.19	0.01	0.24	0.03	-	-	0.27	0.02
C20:4n-3	0.45	0.01	1.61	0.56	0.70	0.02	0.91	0.09
C20:5n-3 (EPA)	7.72	0.79	6.09	0.13	3.52	0.00	2.96	0.34
C22:3	0.59	0.11	0.71	0.27	1.10	0.28	0.41	0.05
C22:5n-3	4.46	0.12	4.51	0.51	2.76	0.02	2.94	0.30
C22:6n-3 (DHA)	17.36	1.98	10.11	1.16	6.85	0.25	9.40	1.45
Total % ID	97.18		99.90		94.86		94.09	

Table A.3. Complete fatty acid profiles of flame angelfish broodstock diets tested in Chapter 6 (Exp. 1). Values are expressed as % of total fat.

Saturates	High n-3 Diet	Med n-3 Diet	Low n-3 Diet	Control Diet
C4:0				0.267
C6:0	0.011	0.012		0.020
C8:0	0.074	0.067	0.044	0.013
C10:0	0.268	0.201	0.154	0.039
C12:0	1.220	0.994	0.756	0.153
C13:0		0.020		0.021
C14:0	8.668	6.297	3.366	3.556
C15:0	0.405	0.284	0.087	0.379
C16:0	21.509	20.214	18.618	24.351
C17:0	0.403	0.327	0.196	0.494
C18:0	4.174	4.032	3.925	5.860
C20:0	0.269	0.327	0.384	0.261
C21:0	0.034	0.026	0.028	0.050
C22:0	0.328	0.279	0.236	0.244
C23:0	0.044	0.044	0.038	0.268
C24:0	0.225	0.180	0.141	0.130
Total Saturates	37.632	33.304	27.973	36.103
Monounsaturates	High n-3 Diet	Med n-3 Diet	Low n-3 Diet	Control Diet
C14:1	0.062	0.061	0.035	0.051
C15:1				
C16:1	6.953	4.860	2.003	3.191
C17:1	0.020	0.020	0.024	0.000
C18:1n-9	17.343	28.592	44.244	15.324
C20:1	1.261	1.026	0.841	1.864
C22:1	0.295	0.227	0.189	0.204
C24:1	0.444	0.411	0.340	0.137
Total Monounsaturates	26.378	35.197	47.676	20.770
Polyunsaturates	High n-3 Diet	Med n-3 Diet	Low n-3 Diet	Control Diet
C18:2n-6	7.624	8.480	9.688	11.436
C18:3n-4	1.457	1.321	1.091	0.114
C18:3n-3	1.194	1.071	0.909	3.763
C20:2n-6	0.274	0.197	0.073	0.575
C20:3n-6	0.258	0.178	0.094	0.177
C20:3n-3	0.206	0.116	0.073	0.236
C20:4n-6 (ARA)	1.494	1.218	0.698	1.331
C20:5n-3 (EPA)	7.136	5.417	2.670	11.324
C22:2	0.044	0.041		0.033
C22:3	0.043	0.045	0.024	0.057
C22:4	0.054	0.034	0.013	0.096
C22:5	1.053	0.745	0.261	0.812
C22:6n-3 (DHA)	15.155	12.635	8.757	13.205
Total Polyunsaturates	35.992	31.498	24.351	43.154

Table A.4. Complete fatty acid profiles of flame angelfish broodstock diets tested in Chapter 6 (Exp. 2). Values are expressed as % of total fat.

Saturates	High 2 Diet	Med 2 Diet	Low 2 Diet	Control Diet
C4:0				0.267
C6:0		0.008	0.005	0.020
C8:0	0.022	0.023	0.015	0.013
C10:0	0.124	0.110	0.095	0.039
C12:0	0.767	0.672	0.544	0.153
C13:0	0.027	0.021	0.019	0.021
C14:0	7.768	6.134	4.683	3.556
C15:0	0.483	0.349	0.252	0.379
C16:0	20.697	19.480	18.210	24.351
C17:0	0.444	0.365	0.284	0.494
C18:0	4.270	4.119	4.048	5.860
C20:0	0.256	0.308	0.375	0.261
C21:0	0.023	0.030	0.009	0.050
C22:0	0.298	0.255	0.254	0.244
C23:0	0.037	0.050	0.051	0.268
C24:0	0.209	0.173	0.156	0.130
Total Saturates	35.425	32.097	29.000	36.103
Monounsaturates	High 2 Diet	Med 2 Diet	Low 2 Diet	Control Diet
C14:1	0.062	0.041	0.044	0.051
C15:1				
C16:1	6.937	5.456	4.174	3.191
C17:1	0.012	0.019	0.013	0.000
C18:1n-9	19.544	29.255	38.185	15.324
C20:1	1.328	1.144	1.033	1.864
C22:1	0.021	0.179	0.142	0.204
C24:1	0.392	0.283	0.233	0.137
Total Monounsaturates	28.296	36.377	43.824	20.770
Polyunsaturates	High 2 Diet	Med 2 Diet	Low 2 Diet	Control Diet
C18:2n-6	6.635	7.517	8.113	11.436
C18:3n-4	0.977	0.911	0.820	0.114
C18:3n-3	1.347	1.216	1.072	3.763
C20:2n-6	0.328	0.246	0.193	0.575
C20:3n-6	0.247	0.181	0.140	0.177
C20:3n-3	0.247	0.183	0.134	0.236
C20:4n-6 (ARA)	1.644	1.443	1.376	1.331
C20:5n-3 (EPA)	7.190	5.751	4.410	11.324
C22:2	0.026	0.021	0.010	0.033
C22:3	0.043	0.036		0.057
C22:4	0.092	0.057	0.072	0.096
C22:5	1.310	0.985	0.720	0.812
C22:6n-3 (DHA)	15.735	12.981	10.116	13.205
Total Polyunsaturates	35.821	31.528	27.176	43.154

BIOGRAPHY OF THE AUTHOR

Chatham Callan was born in northern New Jersey on October 6, 1975. He grew up in Ringwood, New Jersey, and graduated from Hawthorne Christian Academy in 1993. He attended Fairleigh Dickinson University and graduated with a Bachelor of Science degree in Marine Biology in 1997. While working towards his undergraduate degree, he also spent time studying at the University of Hawaii and Cornell University's Shoals Marine Laboratory. Since he was young, he was always interested in tropical fish and maintained several aquariums throughout his childhood and into his adult life. During college, he became the manager of a very large aquarium store in New York and also spent time volunteering at the New York Aquarium.

In 1998, he pursued his interest in aquaculture, and graduated with a Masters degree in Marine Biology from The University of Maine in December 2000. His Masters Thesis research focused on the development of microparticulate diets for rearing Atlantic cod larvae. After receiving his Masters degree, Chatham pursued a career in the research and development of culturing marine ornamental fish species and was employed by Mangrove Tropicals Inc., in Kahuku, Hawaii. He is currently employed as a research associate with the Oceanic Institute in Hawaii. Chatham is a candidate for the Doctor of Philosophy degree in Marine Biology from The University of Maine in August 2007.